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REVIEW ARTICLE

Gastric and Enterohepatic Non-*Helicobacter pylori* Helicobacters

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Abstract

A substantial number of reports published in the last year have contributed to a better understanding of both human and animal infection with non-*Helicobacter pylori Helicobacter* species (NHPH). Gastric infection of humans with *Helicobacter suis* and *Helicobacter felis* as well as unidentified NHPH has been described to cause a chronic gastritis and a variety of clinical symptoms, whereas enterohepatic NHPH, including *Helicobacter cinaedi, Helicobacter bilis,* and *Helicobacter canis,* have been reported to be associated with human diseases such as bacteremia, cellulitis, cutaneous diseases, and fever of unknown origin in immunocompromised hosts. In various animal species, including dogs and laboratory mice, high rates of infection with NHPH were described. For gastric NHPH, mainly *H. suis* and *H. felis* infection was studied, revealing that differences in the immune response evoked in the host do exist when compared to *Helicobacter pylori*. Pathogenic mechanisms of infection with *Helicobacter pullorum, H. bilis,* and *Helicobacter hepaticus* were investigated, as well as immune responses involved in *H. bilis-, Helicobacter typhlonius-,* and *H. hepaticus*-induced intestinal inflammation. Complete genome sequences of *Helicobacter heilmannii* strain ASB1 and a *H. cinaedi* strain isolated in a case of human bacteremia were published, as well as comparative genomics of a human-derived *Helicobacter bizzozeronii* strain and proteome or secretome analyses for *H. hepaticus* and *Helicobacter trogontum,* respectively. Molecular analysis has revealed a function for type VI secretion systems of *H. hepaticus* and *H. pullorum,* the *Helicobacter mustelae* iron urease, and several other functional components of NHPH. In each section of this chapter, new findings on gastric NHPH will first be discussed, followed by those on enterohepatic *Helicobacter* species.

Gastric and Enterohepatic Non-*Helicobacter pylori Helicobacter* (NHPH) Infections in Humans

Several reports describe the association between gastric non-*H. pylori Helicobacter* (NHPH) infections and gastric complaints in human patients. A 17-year-old man suffering from heartburn was diagnosed with gastric NHPH infection, without further species identification. Histopathologic examination revealed a chronic active gastritis as well as lymphoepithelial lesions [1]. Another study aimed at evaluating the incidence of gastric NHPH infection in dyspeptic Polish children (4–18 years of age) [2]. A prevalence of 0.2% was assessed and histopathology showed that most children suffered from a nodular chronic gastritis, which was active in half of the cases and sometimes accompanied by the presence of gastric or duodenal ulcers. No clear association with animal contact was found in this study, in contrast to another report describing the presence of *Helicobacter suis* in a pig veterinarian suffering from general dyspeptic symptoms, reflux esophagitis, and histologically

confirmed chronic gastritis [3]. A German study described the first isolation of *Helicobacter felis* from an infected human, in this case a 14-year-old girl presenting with persistent epigastric pain and vomiting episodes [4]. The authors succeeded by using their routine *Helicobacter pylori* isolation protocol. In general, gastric NHPH infections in the patients described in these studies were successfully eradicated using a triple therapy of at least 1 week, consisting of omeprazole or pantoprazole or bismuth salts, amoxicillin, and clarithromycin or metronidazole. For the *H. suis*-infected pig veterinarian, a mild chronic gastritis was observed upon follow-up gastroscopy [3].

Helicobacter cinaedi has been reported to be associated with bacteremia and cellulitis in an asplenic patient [5] and a patient with systemic lupus erythematosus (SLE) [6]. Forty-seven cases of *H. cinaedi* bacteremia experienced at a hospital as nosocomial infection were evaluated retrospectively [7], and 16 cases (34%) showed cutaneous lesions, indicating that the skin lesions can be an early clinical indicator of *H. cinaedi* bacteremia in the setting of nosocomial infection. Bacteremia cases caused by *Helicobacter bilis* [8] and *Helicobacter canis* [9] were also reported in patients with X-linked agammaglobulinemia (XLA) and common variable immune deficiency, respectively. Antibiotic treatments with ertapenem, azithromycin, levofloxacin, doxycycline, and ceftriaxone were effective in these patients [8,9].

Three studies on the diagnosis of *H. cinaedi* bacteremia were reported by Japanese investigators. Oyama et al. [10] reported a nested PCR assay that rapidly detects the cytolethal distending toxin (*cdt*) gene of *H. cinaedi* with high specificity and sensitivity. This PCR assay was able to identify *H. cinaedi* in blood, urine, and stool samples from a patient with a suspected *H. cinaedi* infection and three patients with known infection. In addition, *H. cinaedi* was detected in stools of 4 of 30 healthy volunteers, suggesting *H. cinaedi* colonization of the intestinal tract. Tomida et al. [11] established a broth microdilution method for antimicrobial susceptibility testing of Japanese clinical *H. cinaedi* isolates and reported that this broth microdilution method was suitable and reliable for antimicrobial susceptibility testing. Rimbara et al. [12] reported the development of a genotyping method, involving multilocus sequence typing (MLST) of 50 *H. cinaedi* strains isolated from 7 Japanese hospitals. Following a comparison of 21 housekeeping genes from 8 *H. cinaedi* isolates, seven genes were selected for MLST, revealing 14 sequence types (STs). It was shown that the isolates from three hospitals belonged to the same STs, whereas the isolates from the other four hospitals belonged to different STs.

Zhou et al. [13] reported a meta-analysis of 10 studies performed between 2002 and 2011 that aimed at investigating an association between *Helicobacter* spp. infection in the biliary system and biliary tract cancer. A much higher prevalence rate of *H. pylori* infection was observed in the malignant group compared to the benign biliary disease group in six studies. Similarly, the pooled prevalence of *H. bilis* infection in four studies was significantly higher in the malignant group. Analysis for two other species (*Helicobacter hepaticus* and *Helicobacter ganmani*), however, did not reveal differences between the two above-mentioned groups.

Infection with Gastric and Enterohepatic NHPH in Animals

In a study by Ekman et al. [14], a high prevalence for *H. canis*, *Helicobacter bizzozeronii*, and *Helicobacter salomonis* was observed in clinically healthy Beagle dogs. *H. canis* was detected in the feces and saliva of a large portion of the animals, suggesting that this enterohepatic *Helicobacter* species may be transmitted via the fecal/anal–oral route. For *H. bizzozeronii* and *H. salomonis*, bacteria or DNA were mainly detected in the stomach and duodenum and occasionally in saliva. The prevalence of a third gastric *Helicobacter* species, *H. felis*, was lower, and bacteria were only detected in stomachs. All three gastric *Helicobacter* species could not be detected from the feces of these dogs. In another study, the ^{13}C urea breath test was shown to be useful for the detection of gastric Helicobacters in dogs, with the authors reporting a sensitivity and specificity of 89% [15].

For the first time, infection with a pure *in vitro* isolated strain of *H. suis* was performed in pigs [16]. This study showed a clear causal relationship between experimental *H. suis* infection in pigs and the development of a chronic gastritis, as well as a 10% decrease in the daily weight gain of the animals. In addition, a tendency toward the development of more severe hyperkeratosis, erosion, and ulceration of the pars esophagea of the stomach was observed in infected animals.

Cacioppo et al. [17] reported the infectivity of *Helicobacter pullorum* in brown Norway rats. At 4 weeks postinfection (pi) and continuing throughout 30 weeks pi, *H. pullorum* was detected by PCR in 5 of 6 rats, but no significant lesions in the intestine were observed in the experimentally infected rats. In contrast, the same group [18] reported that Sprague-Dawley rats were resistant to infection with *H. pullorum*. The reason why the colonization of *H. pullorum* was different between Sprague-Dawley rats and brown Norway rats remains

to be determined, and further epidemiologic studies of *H. pullorum* are warranted.

Several reports have addressed the prevalence and natural occurrence of enterohepatic NHPH in animals. Lofgren et al. [19] investigated the prevalence of murine enterohepatic NHPH in six animal facilities in 2009. The mean prevalence of NHPH in these six facilities was 28%; the prevalence of *H. hepaticus, H. bilis, H. rodentium, H. typhlonicus,* and *H. mastomyrinus* were 26, 16, 12, 16, and 4%, respectively. Four of the six facilities surveyed in 2009 were also surveyed 10 years before. Over this time period, there was a substantial reduction in *H. rodentium* infection from 76 to 12%, a moderate decrease in *H. hepaticus* infection from 41 to 26%, and a small increase in the *H. bilis* prevalence from 6 to 16% in these mouse facilities. Wasimuddin et al. [20] reported that the most frequently detected enterohepatic NHPH species in wild house mice were *H. rodentium* (78%), *Helicobacter typhlonius* (53%), *H. hepaticus* (41%), and *H. bilis* (30%). Therefore, it should be considered that wild house mice can be potential reservoirs of enterohepatic NHPH infection for both humans and other vertebrates. Nam et al. reported that 103 C3H/HeNrs mice among 978 suffered from mild to severe chronic hepatitis, regardless of irradiation exposure. Helical-shaped bacteria were detected between hepatocytes by silver staining and immunohistochemistry, suggesting that the hepatitis was caused by *Helicobacter* spp. [21] Unidentified *Helicobacter* spp. (strain MIT01-6451) were also detected in all cecum, colon, and feces samples of 167 specific-pathogen-free mice in Japan [22]. Finally, Hayashimoto et al. [23] reported that the most frequently detected microbe in mouse and rat facilities was indeed *Staphylococcus aureus* (18.8% in 3459 mouse facilities, 58.6% in 772 rat facilities). *H. hepaticus* and *H. bilis* were detected by PCR in 3.17% and 0.17% of these same mouse facilities, harboring more than 14,000 mice. These results indicate that monitoring the presence of enterohepatic NHPH is very important for quality control of animal experiments.

Immune Response Against Gastric NHPH and Pathogenesis of Infection

Helicobacter felis infection in mice is often used as an animal model to study *H. pylori*-related gastric pathology in humans. Researchers must be aware that *Helicobacter*-induced gastritis and carcinogenesis in mouse models may very well be augmented or attenuated by several other infectious agents, probably via the immune response [24].

Hitzler et al. [25] showed that caspase-$1^{-/-}$ mice were able to control an *H. felis* infection more efficiently compared to wild-type mice, although the role of caspase-1 in *Helicobacter* sp. infection is indeed dual, possessing both regulatory and pro-inflammatory properties. Another study by the same research group, using knockout mice on a C57BL/6 background [26], demonstrated that a functional T-cell receptor is essential for the control of an *H. felis* infection as well as for the induction of gastric neoplastic pathology. The authors demonstrated that neither IL-12-dependent Th1 nor IL-23-dependent Th17 cells are indispensable to control *H. felis* and *H. pylori* infection. Only for the latter, the absence of Th17-polarizing IL-23 resulted in less gastritis and less precancerous lesions. These results are somewhat surprising, as both Th1 and Th17 cell subsets are crucial for vaccination-induced protection against gastric Helicobacters. This was confirmed in an *H. suis* mouse experiment, showing that immunization of mice with *H. suis* lysate as well as the *H. suis* ureB subunit was capable of inducing a significant reduction in the bacterial load [27], which indeed was shown to depend on local Th1 and Th17 responses, as well as the decreased expression of regulatory IL-10. The choice of antigen in this study was based on an immunoproteomics study, revealing a pronounced immunoreactivity in *H. suis* lysate-immunized mice against several proteins, including UreB.

Although gastric NHPH infections, especially with *H. felis,* are often used to study the host immune response to *H. pylori,* data need to be interpreted with caution. A study by Flahou et al. [28] showed that experimental *H. suis* infection in WT BALB/c and C57BL/6 mice causes an upregulation of IL-17 and IL-10 expression, which resembles *H. pylori* infection. On the other hand, most *H. suis* strains caused an upregulation of IL-4 expression (a Th2 cytokine), and increased levels of IFN-γ mRNA were never detected, which is clearly distinct from the immune response elicited by *H. pylori* infection in this animal model. These differences might be responsible for the increased risk of developing gastric MALT lymphoma in human patients infected with gastric NHPH. A Japanese study showed that overexpression of *miR-142-5p* and *miR-155* is induced by NHPH infection in C57BL/6 mice, which is an animal model of gastric MALT lymphoma [29]. The authors hypothesize that these miRNAs might serve as novel biomarkers for gastric MALT lymphoma.

A long-term *H. felis* infection study in transgenic mice carrying the human IL-8 gene and its regulatory elements [30] showed that IL-8 upregulation during *H. felis* infection is associated with an increase in pseudopyloric metaplasia and dysplasia. The increased mobilization of immature $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells may be involved in the development of these precancerous

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lesions. In *H. felis*-infected mice, spasmolytic polypeptide-expressing metaplasia (SPEM) develops as another preneoplastic lesion after parietal cell loss, and Weis et al. [31] showed that clusterin serves as a clear marker of all SPEM lineages in mice and humans, whereas cystic fibrosis transmembrane conductance regulator (CFTR) was upregulated only in SPEM with inflammation in mice, revealing a clear heterogeneity of phenotypic metaplastic lineages.

Inflammation-related changes induced by gastric NHPH were not the only ones investigated in the past year. Baird et al. [32] demonstrated the importance of a sustained induction of the unfolded protein response (UPR) in a mouse model of gastric cancer, as shown by the increased expression of the endoplasmic reticulum stress marker HSPA5 in the metaplastic region of WT C57BL/6 mice infected with *H. felis* for 78 weeks. Other experiments describing *H. felis* infection in wild-type, knockout, and transgenic C57BL/6 mice revealed that CD24, expressed in gastric parietal cells, modulates colonization rates and gastric responses (inflammation, atrophy) to *H. felis* infection [33] and that *H. felis* infection increases the gastric abundance of plasminogen activator inhibitor (PAI)-1, which is associated with resistance to the satiating effects of CCK8 [34]. Finally, a clear association between decreased serum iron concentrations and parietal cell loss and concomitant hypochlorhydria was found in *H. felis*-infected INS-GAS mice, in which altered gastric expression of iron metabolism regulators/transporters was observed [35].

Immune Response Against Enterohepatic NHPH and Pathogenesis of Infection

Regulation of intestinal inflammation mediated by IL-7R (receptor)$^+$ innate lymphoid cells (ILCs) was reported by Powell et al. By comparing intestinal microbiota between TRUC (*Tbx21*$^{-/-}$*Rag2*$^{-/-}$ ulcerative colitis) mice and TRnUC (*Tbx21*$^{-/-}$*Rag2*$^{-/-}$ nonulcerative colitis) mice, *H. typhlonius* was identified as a key disease trigger, driving excess TNF-α production and promoting colitis. It was shown that oral inoculation with *Helicobacter trogontum* resulted in an increased abundance of *Tnfa* transcripts in the colon of TRnUC mice to levels similar to those observed in TRUC mice. It was also demonstrated that specific IL-7R blockades significantly diminished colonic ILCs and suppressed colitis [36]. The role of macrophages in *H. bilis* -induced proinflammatory cytokine-mediated typhlocolitis was examined by using BALB/c *Rag2*$^{-/-}$ mice lacking functional lymphocytes in which clodronate (a macrophage depleting drug) was administered. At 16 weeks pi, the ceca of *H. bilis* -infected *Rag2*$^{-/-}$ mice treated with control liposomes showed significantly higher histopathologic scores for typhlocolitis and higher counts of macrophages and myeloperoxidase-positive neutrophils compared to *H. bilis* -infected *Rag2*$^{-/-}$ mice treated with clodronate-coated liposomes, suggesting that macrophages are critical inflammatory cellular mediators [37]. McCaskey et al. [38] reported that SMAD3$^{-/-}$ mice, but not SMAD3$^{-/+}$ mice, developed colitis following *H. hepaticus* infection. CD4$^+$ and CD8$^+$/CD62L^{lo} cells, an effector T lymphocyte population, as well as NK cells were significantly higher in the mesenteric lymph nodes of SMAD3$^{-/-}$ mice. The obtained results suggest that defects in SMAD3 signaling increase the susceptibility to *H. hepaticus* -induced colitis through aberrant activation and/or dysregulation of effector lymphocytes. Morrison et al. [39] reported that intestinal inflammation triggered by *H. hepaticus* correlated with elevated frequencies and numbers of lamina propria CD4$^+$ T cells expressing IFN-γ or IFN-γ plus IL-17A. It was also demonstrated that IL-17A$^+$ lymphocytes arising after *H. hepaticus* inoculation extinguish their IL-17A secretion and switch phenotype to IFN-γ$^+$ ex-Th17 cells.

Several studies on the pathogenesis of enterohepatic NHPH infection were reported. Sirianni et al. [40] reported that *H. pullorum* can adhere to and invade human intestinal Caco-2 cells. Thirty-three of 137 identified proteins were bioinformatically predicted to be secreted. Okoli et al. [41] compared *H. bilis* -associated protein expression in human hepatoma Huh7 cells harboring a replicon of hepatitis type C virus (HCV) and in the replicon-cured cells. In the transfected Huh7 cells inoculated with *H. bilis*, 53 different proteins were identified using differential protein expression analysis, and 44 proteins were identified in the cured cells inoculated with *H. bilis*. Le Roux-Goglin et al. [42] observed hepatic lesions in hepatitis C virus (HCV) transgenic mice infected with *H. hepaticus*. The authors found that *H. hepaticus* infection, but not the HCV transgene, increased the number of hepatic lesions. It was concluded that the synergism between HCV and *H. hepaticus* infection involved in liver disease may be highly host dependent.

Zhang et al. investigated the effect of probiotic *Lactobacillus acidophilus* strains on the growth of *H. hepaticus* [43]. Supernatants of *L. acidophilus* significantly reduced the cell growth rate and the urease activity of *H. hepaticus* in a time-dependent manner, and the inhibitory effect was shown to be independent of the pH value of the solution. The results provide evidence for developing novel approaches for the prevention and treatment of *H. hepaticus* infection.

Genomics, Evolution, and Strain Typing

The complete genome sequence of *Helicobacter heilmannii* strain ASB1 was determined, revealing the presence of various genes encoding homologs of known *H. pylori* virulence factors, such as the GGT, NapA, HtrA, but also the absence of others, including Bab and Sab adhesins, VacA and the *cag* pathogenicity island (PAI) [44]. When mapped against a corpus-derived reference *H. bizzozeronii* genome, comparative genomics of antrum-derived *H. bizzozeronii* in a human stomach before and after unsuccessful eradication revealed a certain level of allelic variability before treatment and the emergence of a new antral population after unsuccessful treatment, characterized by an increased number of fixed and new mutations [45].

A multilocus sequence typing method was developed for *H. suis*, revealing that *H. suis* is a genetically diverse bacterial species on the pig herd level [46]. In addition, strain typing revealed that the *H. suis* strain colonizing the pig veterinarian described above [3] showed a very close relationship to porcine *H. suis* strains.

Moodley et al. [47] described how the *H. pylori* phylogeny splits into 2 primary superlineages, after which the closely related *H. acinonychis* originated from a host jump from the San people to large felines approximately 43,000–56,000 years ago.

The complete genome sequence of *H. cinaedi* strain PAGU611 isolated in a case of human bacteremia was reported [48]. The PAGU611 genome is comprised of a 2,078,348-bp chromosome and a 23,054-bp plasmid (pHci1) with average G+C contents of 38.6% and 31.6%, respectively. Synteny plots identified a unique *H. cinaedi* genomic island (HciGI1) containing 173 protein-coding sequences including 147 hypothetical protein genes and 12 genes to assemble a type VI secretion system (T6SS). Okoli et al. [49] reported the effects of human and porcine bile on the proteome of *H. hepaticus*, revealing that 46 proteins of *H. hepaticus* were differentially expressed in human bile, and 32 proteins were differentially expressed in porcine bile. These data suggest that bile is an important factor that determines the virulence, host adaptation, localization, and colonization of specific niches within the host environment. Kaakoush et al. [50] identified 104 proteins of *H. trogontum* that were bioinformatically predicted to be secreted, including 11, 11, 3, and 3 proteins involved in the response to oxidative stress or redox reactions, motility, virulence, and the T6SS, respectively.

Molecular Biology

An apoprotein form of the *Helicobacter mustelae* iron urease, encoded by *ureA2B2* genes, was shown to be activated with ferrous ions in the absence of auxiliary proteins, but not with nickel ions, as goes for the "standard" gastric *Helicobacter* ureAB, which also needs accessory proteins for its proper activity [51]. Schur et al. [52] cloned and expressed HAC1267 and HAC1268, 2 sialyltransferase enzymes of the GT-42 family from *H. acinonychis* strain ATCC 51104, revealing that HAC1268 is the first member of this family showing α2,6-sialyltransferase activity.

The construction and characterization of a *nik*R mutant strain of *H. hepaticus* was reported [53]. Disruption of this gene, encoding the nickel-responsive regulator NikR, led to increased activities of two Ni-requiring enzymes: urease and hydrogenase. In addition, the mutant strain had a two- to threefold lower growth yield than the wild-type strain, suggesting that the regulatory protein might play additional roles in this mouse liver pathogen. Bartonickova et al. [54] characterized the *H. hepaticus* T6SS components Hcp, VgrG1, VgrG2, and VgrG3 in a C57BL/6 Rag2$^{-/-}$ mouse T-cell transfer model. Transcripts of all *hcp* and *vrg*G genes were detected upon growth of the bacteria under *in vitro* and *in vivo* conditions. The *vgr*G1 mutant-infected T-cell-transferred mice showed only slight or no macroscopically visible pathologic aspects in both the cecum and the colon. It was also shown that cellular innate pro-inflammatory responses were increased by the secreted VgrG1 and VgrG2.

HP1043 of *H. pylori* is an orphan response regulator (RR) with a highly degenerate receiver sequence incapable of phosphorylation. In a report by Bauer et al. [55], the *H. pullorum* two-component system (TCS), consisting of the HP1043 ortholog HPMG439 and its cognate histidine kinase (HK) HPMG440, was characterized. It was demonstrated that the consensus RR HPMG439 of *H. pullorum* can functionally replace the atypical HP1043 protein in *H. pylori*. This TCS was shown to be involved in the control of nitrogen metabolism by regulating the expression of glutamate dehydrogenase, an AmtB ammonium transporter and a P_{II} protein.

Conclusion

Over the past 12 months, significant advances have been made in research on both gastric and enterohepatic NHPH species, especially with regard to the pathogenesis of infection and the immune response these

bacteria induce in their hosts. It was clearly shown that the importance of infection with NHPH in humans as well as animals should not be neglected.

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Impact of *Helicobacter pylori* Biofilm Formation on Clarithromycin Susceptibility and Generation of Resistance Mutations

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Abstract

The human gastric pathogen *Helicobacter pylori* forms biofilms *in vitro* and *in vivo*. The purpose of this study was to evaluate the effects of *H. pylori* biofilm formation *in vitro* on clarithromycin (CLR) susceptibility. CLR susceptibility of *H. pylori* intermediate (2-day) and mature (3-day) biofilms on glass coverslips was determined at concentrations from 0.03 to 0.5 μg/ml. *H. pylori* biofilm biomass was increased after treatment with CLR at minimum inhibitory concentration levels by up to 4-fold (2-day biofilm) and 16-fold (3-day biofilm). Minimum bactericidal concentrations of CLR against cells in a biofilm were higher (1.0 μg/ml) than that for planktonic cells (0.25 μg/ml). It was shown that the expression of efflux pump genes was significantly increased in biofilm cells. In addition, exposure of biofilms to CLR resulted in high level resistance generation compared to planktonic cells with increased resistance associated with the presence of a point mutation at either position 2142 or 2143 in the domain V loop of the 23S rRNA gene. These results demonstrate that *H. pylori* biofilm formation decreases the susceptibility to CLR and that *H. pylori* CLR resistance mutations are more frequently generated in biofilms than in planktonic cells.

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Introduction

Biofilms are ubiquitous in natural, industrial and clinical environments, and have been shown to be critical in many chronic infections [1]. Biofilm development is initiated when bacteria convert from a planktonic state to a lifestyle in which they are firmly attached to biotic or abiotic surfaces. Biofilm bacteria express several properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents and this process is thought to be a major contributor to the etiology of infectious diseases [2].

Helicobacter pylori is a spiral, microaerophilic, non-invasive, Gram-negative bacterium that colonizes the human gastrointestinal tract, primarily the stomach [3]. Recently, some studies have alluded to the ability of *H. pylori* to form biofilms *in vitro* [4,5,6]. In addition, *H. pylori* can exist in biofilms formed on human gastric mucosa [7,8,9]. Our previous study demonstrated that the strain TK1402, isolated from a patient with duodenal and gastric ulcers in Japan had strong biofilm forming ability *in vitro* [10,11,12].

As the first-line therapy for *H. pylori* eradication, the combination of a proton pump inhibitor, either clarithromycin (CLR) or metronidazole, and amoxicillin has been established worldwide [13,14]. In Japan, a combination of a proton pump inhibitor, amoxicillin and CLR is commonly used in first-line eradication therapy [15]. CLR is a macrolide antibiotic that binds to the 50S subunit of the bacterial ribosome and inhibits the translation of peptides, thus preventing the bacteria from growing. However, CLR-resistance is an increasing problem for the first-line therapy of *H. pylori* infection, since the major cause of eradication failure is thought to be the existence of CLR-resistant *H. pylori* [14–18]. CLR resistant *H. pylori* are extremely common and the frequency of CLR-resistant clinical isolates ranges from approximately 10 to 30% [19,20]. Point mutations in the domain V loop of the 23S rRNA gene (commonly an adenine-to-guanine transition at position 2142 or 2143) have been reported as the basis for resistance [15–23].

The aim of this study was to investigate the effects of CLR on *H. pylori* biofilms *in vitro*. Furthermore, we compared the generation of spontaneous resistance to various concentrations of CLR in both biofilm and planktonic cells and the mutations in the 23S rRNA gene of *H. pylori* were determined.

Materials and Methods

Bacterial Strain

H. pylori strain TK1402, isolated from gastric biopsy specimens of a patient with gastric and duodenal ulcers [24], was used in this study. The strain was maintained at −80°C in Brucella broth (Difco, Detroit, Mich) with 20% (vol/vol) glycerol and was cultured under microaerobic conditions at 37°C on Brucella agar plates containing 7% horse serum.

Assessment of Susceptibility to CLR

H. pylori strain TK1402 was grown on Brucella agar plates containing 7% horse serum; the cells were then suspended in fresh Brucella broth supplemented with 7% fetal calf serum (Brucella-FCS) and cultured for 24 h under microaerobic conditions at 37°C. The pre-culture cells were adjusted to an optical density of 1.0 at 600 nm. In a 12-well microtiter plate, 10 µl of pre-cultured cells were inoculated into 2 ml of duplicate serial two-fold dilutions of CLR in Brucella-FCS from 0.001 µg/ml to 1.0 µg/ml. The cultures were incubated under microaerobic conditions at 37°C for 24 h with shaking (80–100 rpm). After incubation, optical densities of the cultures were examined as the means of three independent experiments.

Assessment of biofilm cell susceptibility to CLR was carried out by a similar method as above using 2-day and 3- day biofilms with slight modifications. Briefly, biofilms of *H. pylori* strain TK1402 were grown as previously described [10]. The coverslips with 2-day or 3-day biofilms were removed from the 12-well plate, washed with phosphate-buffered saline (PBS) and placed into fresh plate wells filled with 2 ml Brucella-FCS and 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, 0.063 µg/ml, 0.031 µg/ml, or 0 µg/ml of CLR. The biofilm cells were then incubated for 24 h under microaerobic conditions at 37°C with shaking. After incubation, the biofilms were assessed with the biofilm assay previously described [10].

Determination of Cell Viability

To determine the number of viable cells after exposure to CLR, 2-day or 3-day biofilm coverslips were transferred to 2 ml of fresh Brucella-FCS with two-fold dilutions of CLR at concentrations ranging from 0.03 µg/ml to 0.5 µg/ml. Two-day or 3-day planktonic cultures of cells exposed to the same concentrations of CLR were used as controls. After exposure, the biofilm cells were scraped and removed with PBS following mechanical treatment. The colony-forming unit (CFU) values of the cell suspension was then determinbed by plating onto Brucella agar supplemented with 7% FCS (Brucella-FCS agar). The CFUs of the 2-day or 3-day planktonic cell cultures were also evaluated using the same method. Colony numbers were counted after 96 h incubation.

To confirm the cell morphology after treatment with CLR, a scanning electron microscope (SEM) examination was carried out. The CLR exposed 3-day biofilms on the coverslips were fixed with 2% glutaraldehyde for 3 h at room temperature. The samples were observed using a JSM-5600LV electron microscope (JEOL, Tokyo, Japan).

Extraction of RNA and Real-time Quantitative RT-PCR

To analyze the expression of mRNA in the *H. pylori* biofilms, the 3-day biofilm cells of strain TK1402 were scraped into PBS. Planktonic cells were also cultivated for 3 days at 37°C. After washing of the cells with PBS three times, total RNA extraction was carried out using the RNeasy minikit (QIAGEN GmbH, Hilden, Germany). The RNA samples were then treated with a TURBO DNA-free™ Kit (Applied Biosystems, Foster City, CA) according to the directions of the supplier. Reverse transcription (RT) was carried out with the PrimeScript™ RT reagent Kit (Takara Bio INC. Shiga, Japan) according to the directions of the supplier. Real-time RT-PCR was performed with the cDNA samples with either 16S rRNA-specific primers (Primer Hp16S F; 5′-GAAGATAATGACGGTATCTAAC; R; 5′-ATTTCACACCTGACTAT) [25], or efflux pump-specific primers (HP609 F; 5′-AGCGCAAGAACTCAGTGTCA, R; 5′-GCTTGGAGTTGTTGGGTGTT, HP971 F; 5′-TTACCGGCAAAGGGATACG, R; 5′-AAATTGGATCGCTCGTTGTATG, HP1327 F; 5′-GCCAGGCTTGATGAAGAAAA, R; 5′-TTAGCCTGCTTGCCGTAAAT, or HP1489 F; 5′-TAGGCGCTCAAGTGGCTTAT, R; 5′-TCAGATCGGGCAGATTTTTC) [26], with the SYBR^{R} Premix Ex Taq (Perfect Real Time) Kit (Takara Bio INC.) in an ABI PRISM 7500 Real-time PCR system (Applied Biosystems). The final results were expressed as the levels of the expression of each efflux pump gene relative to that of the 16S rRNA gene.

Generation of CLR Resistant Mutants

H. pylori strain TK1402 biofilm cells were subjected to a passage experiment to generate CLR resistance. The coverslips with 2-day or 3-day biofilms were transferred to 2 ml of fresh Brucella-FCS containing CLR at concentrations of 0.125 µg/ml, 0.25 µg/ml or 0.5 µg/ml. The coverslips with the biofilms were then incubated for 24 h under microaerobic conditions at 37°C with shaking. After incubation, the biofilm cells on the coverslips were mechanically scraped, resuspended into PBS, and the cells were recovered following incubation on Brucella-FCS agar plates for 72 h. All of surface growth after 72 h of incubation was transferred with a swab onto antibiotic free agar plates for isolation and also onto plates containing 1.0 µg/ml of CLR to confirm the generation of CLR resistant cells. If no CLR resistant cells were detected, this process was repeated at least 5 times or until thegeneration of CLR resistant cells was detected.

The same method was used for planktonic cells with some modifications. *H. pylori* was cultured for 48 h (2-day culture planktonic cells) or 72 h (3-day culture planktonic cells). Since our previous study noted that the optical density of scraped biofilms were approximately 0.14 or 0.26 for 2-day and 3-day biofilms, respectively [10], the cultures were adjusted to an optical density of 0.14 for 2-day or 0.26 for 3-day planktonic cells in 2 ml of Brucella-FCS containing 0.125 µg/ml or 0.063 µg/ml of CLR in a 12-well microtiter plate. After incubation, the cells were collected by centrifugation and washed with PBS. The cells were recovered and the generation of CLR resistance was determined as described above.

DNA Sequencing of the 23S rRNA Domain

The CLR resistant colonies were picked from CLR free Brucella-FCS agar plates and genomic DNA was extracted using MagExtractor™ (TOYOBO CO., LTD. Osaka, Japan) according to the instructions of the supplier. Genomic DNA served as the template for PCR using a specific primer pair to the domain V loop of the 23S rRNA gene (primer Hp23S 1942F; 5′-AGGATGCGTCAGTCGCAAGAT; Hp23S 2308R; 5′-CCTGTGGATAACACAGGCCAGT) [27]. Nucleotide sequences were analyzed directly from purified PCR products. Sequencing reactions were performed in a BioRad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using ABI BigDye ™ Terminator v3.1 Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems, Foster City, CA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using Hp23S 1942F and Hp23S

2308R primers with an ABI 3730xl sequencer (Applied Biosystems).

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney U test. P values of 0.05 or less were considered to indicate statistical significance.

Results

Susceptibility of Strain TK1402 in Broth Culture to CLR

Since we used broth media for analyzing the CLR susceptibility of biofilm cells in our experimental model, broth microdilution minimum inhibitory concentration (MIC) determinations were carried out using two-fold serial dilutions of the compound with approximately 5×10^5 cells of initial inoculum in 2 ml of Brucella-FCS (Fig. 1). The cells could not grow in broth media at CLR concentrations of 0.03 μg/ml, whereas the cells grew significantly at 0.016 μg/ml of CLR, indicating that the MIC to CLR under this condition is approximately 0.016 μg/ml. The Clinical and Laboratory Standard Institute defined resistance of *H. pylori* isolates to CLR as MIC of >1 μg/ml, indicating that strain TK1402 was susceptible to CLR.

Susceptibility of Strain TK1402 Biofilms to CLR

In order to examine the susceptibility of strain TK1402 biofilms to CLR, the 2-day or 3-day biofilms were exposed to CLR at concentrations ranging from 0.031 μg/ml to 0.5 μg/ml, which are concentrations equivalent to 2×MIC to 32×MIC. In the biofilm assay with strain TK1402, the initial mean absorbance value at 594 nm was approximately 0.533 and 1.511 for 2-day and 3-day biofilms, respectively [10], which were normalized to 1.0. In 2-day biofilms, treatment with 0.063 μg/ml of CLR (i.e. 4×MIC) caused a significant increase in biofilm biomass of approximately 1.2 fold (Fig. 2a). Moreover, the exposure of 3-day biofilms to 0.25 μg/ml CLR (i.e. 16×MIC) significantly increased the biofilm biomass, again by approximately 1.2 fold compared to the initial biofilm (Fig. 2b).

Cell Viability of Biofilm Cells after Treatment of CLR

The results indicating concentration-inversely dependent increase of the biofilm biomass after treatment with CLR might imply that the biofilm cells not only could survive but also grow in the presence of a relatively high concentration of CLR. However, crystal violet staining measures the total biofilm biomass but not cell viability [28]. To clarify whether the increasing biofilm biomass was due to cell growth, cell viability was measured by CFU counting of 2-day biofilms treated with serial dilution of CLR. Similarly treated 2-day planktonic cultures were also analyzed as a control. The CFUs of both the CLR exposed planktonic cultures and biofilms exhibited reduced cell viability (Fig. 3), compared to a slight increase in the untreated control plantonic and biofilm cells. When the cells were exposed to 0.25 μg/ml CLR, no viable colonies were detected in the planktonic cells whereas the biofilm cells could survive with an approximate 10^3 CFU value. In addition, there was significantly increased viable cells in biofilms compared to planktonic cells after treatment with 0.06 μg/ml to 0.5 μg/ml CLR. However, there were no viable colonies when the biofilms were exposed to 1.0 μg/ml CLR. We also examined the CFU value of 3-day biofilms after exposure to CLR and the results were comparable to that of 2-day biofilms (data not shown). Further, we examined the cell morphology after 24 h of CLR treatment using a scanning electron microscope with 3-day biofilm (Fig. 4), since *H. pylori* can transform into a coccoid form, which is non-culturable but viable [29]. The control cells (without treatment of CLR) were composed primarily of cells with bacillary morphology which were clearly outlined (Fig. 4a). On the other hand, when the cells were treated with 0.03 μg/ml (Fig. 4b), 0.06 μg/ml (Fig. 4c) or 0.5 μg/ml (Fig. 4d) of CLR, almost all cells exhibited a rough outline and displayed damaged cell envelopes, although a few coccoid-like forms of cells were detected in the cells of CLR-treated biofilms. These results suggested that most of the cell mass increase in the biofilm represented dead cells. Nevertheless, Fig. 3 indicated that the minimum bactericidal concentration (MBC) of the biofilm cells to CLR was higher than that of planktonic cells (1.0 μg/ml vs. 0.25 μg/ml).

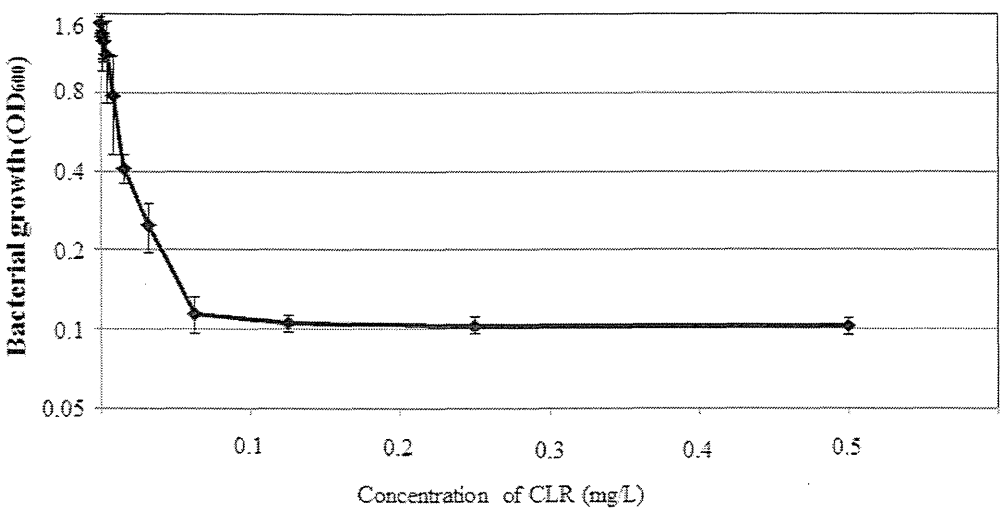

Figure 1. Growth kinetics of *H. pylori* strain TK1402 with CLR. Pre-cultured cells were grown in Brucella-FCS for 24 h with each concentration in a range of 0.5 μg/ml to 0.001 μg/ml or 0 μg/ml of CLR. After incubation for 24 h under microaerobic and shaking condition at 37°C, the optical densities of the cultures were determined. All of the results were expressed as the means ±1 standard deviation from at least three independent experiments.
doi:10.1371/journal.pone.0073301.g001

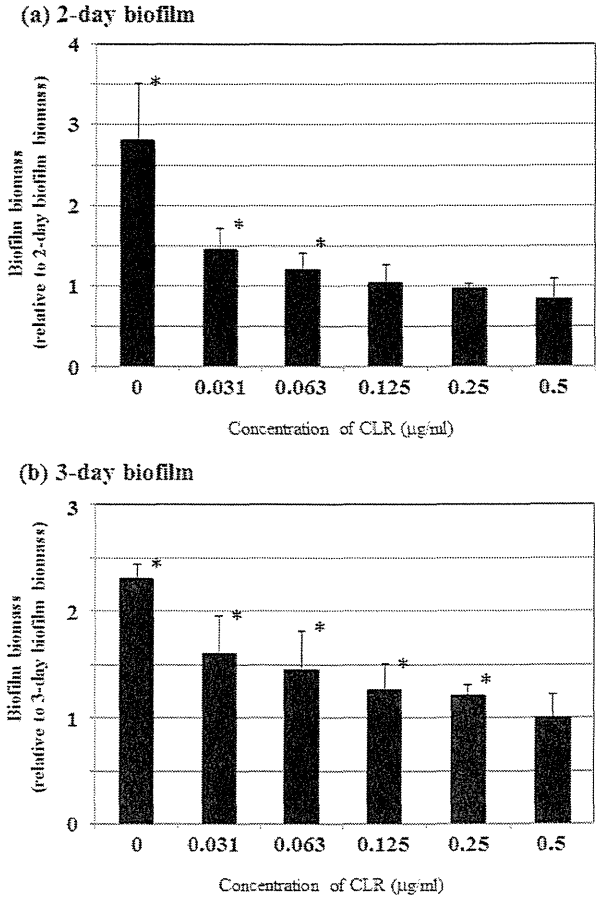

Figure 2. Effect of CLR on strain TK1402 biofilms. The 2-day (a) and 3-day (b) biofilms were transferred into fresh Brucella-FCS with each concentration (0.5 μg/ml, 0.25 μg/ml, 0.125 μg/ml, 0.063 μg/ml, 0.031 μg/ml or 0 μg/ml) of CLR. After incubation for an additional 24 h under microaerobic and shaking conditions at 37°C, the biofilm biomass was measured with crystal violet. The biofilm biomass was calculated relative to starting biofilm biomass (0.53 and 1.51 for 2-day and 3-day biofilm, respectively), which was set at 1.0. All of the results are expressed as the means ±1 standard deviation from at least three independent experiments.*significantly different ($p<0.05$) relative to the level of starting biofilm biomass (starting biofilm biomass versus after biofilm biomass exposure to CLR).
doi:10.1371/journal.pone.0073301.g002

Gene Expression of RND Efflux Pumps in Biofilms

In *H. pylori*, the efflux pumps of the resistance-nodulation-cell division (RND) family are well described relative to their contribution to antibiotic resistance [26,30,31]. These reports indicated that four RND families have been identified in *H. pylori* (HP0605-HP607, HP0971-HP0969, HP1327-HP1329, and HP1489-HP1487). Therefore, we determined whether there were differences in the levels of transcription of these genes between biofilm and planktonic cells using specific primer pairs for HP605, HP971, HP1327, or HP1489 (primers sequences are described in Materials and Methods) with quantitative real-time RT-PCR (Fig. 5). It was revealed that the expression of these genes was significantly more elevated in the biofilm cells than in the planktonic cells. These results suggested that the high levels of these gene transcripts could contribute to biofilm resistance to CLR.

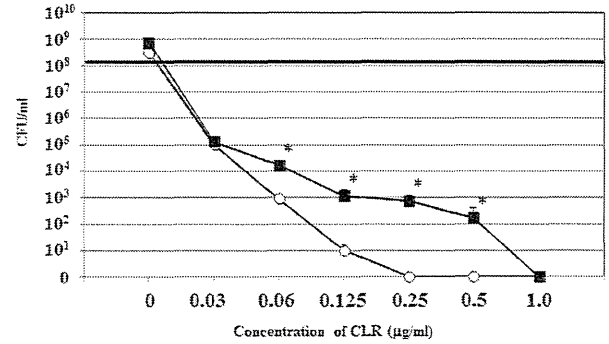

Figure 3. The effect of CLR on cell viability of the strain TK1402 biofilm. After exposure of 2-day biofilm (closed squares) and planktonic cells (open circles) with each concentration of CLR, viable cells were measured using CFU counting. The initial CFU for 2-day biofilm and planktonic cells adjusted to an optical density at 600 nm of 0.14 were approximately 0.3×10^8 CFU. The CFU of CLR exposed biofilm or planktonic cells were measured. All of the results are expressed as the means ±1 standard deviation from at least three independent experiments. *significantly different ($p<0.05$) relative to CFU value (biofilm versus planktonic after treatment with the indicated concentrations of CLR concentration).
doi:10.1371/journal.pone.0073301.g003

Generation of Spontaneous CLR Resistant Mutations in Biofilms

To examine the effect of biofilm formation by *H. pylori* on the generation of spontaneous resistant cells after exposure to CLR, the 2-day or 3-day biofilms were exposed to one-eighth, one-quarter or one-half of the MBC (Fig. 3) of CLR at concentrations of 0.125, 0.25, and 0.5 μg/ml, concentrations which are equivalent to 8×, 16×, and 32×MIC, up to 5 times or until a generation of CLR resistant cells was evident. As controls, 2-day or 3-day planktonic cultures were also exposed to one-quarter or one-half of the MBC (for planktonic cells, as shown in Fig. 3) of CLR at concentrations of 0.063 and 0.125 μg/ml, concentrations which are equivalent to 4× and 8× MIC. Fig. 6 shows the results of the accumulation ratio of the generated CLR resistant biofilm or planktonic cultures. In 2-day planktonic cultures, a few CLR resistant mutants were observed (25% (3/12) and 33% (4/12) at 0.063 μg/ml and 0.125 μg/ml of CLR, respectively) (Fig. 6a). In 3-day planktonic cultures, the generation of resistant cells was at a similar level as in 2-day planktonic cultures (Fig. 6c). In contrast, CLR resistant cells in biofilms were detected more frequently at 0.25 μg/ml (one-quarter MBC) CLR than that in control. Nine of twelve 2-day biofilm cells (75%) were CLR resistant (Fig. 6b), which increased to 84.6% (eleven of thirteen) in 3-day biofilms (Fig. 6d). Additionally, 3-day biofilms showed increased resistance at 0.5 μg/ml or 0.125 μg/ml CLR compared to controls (Fig. 6d), though there was no difference at these concentrations in 2-day biofilms.

Sequence Analysis of 23S rRNA Mutations

All CLR resistant samples (54 samples in total) obtained in this study were examined for 23S rRNA point mutations. The primer pairs used (Hp23S 1942F and Hp23S 2308R), could detect the common mutations (at positions 2142 and 2143 of the 23S rRNA gene) associated with CLR resistance and sequence analysis of the PCR products was then carried out. All of the mutant strains showed a point mutation at either position 2142 (43 strains) or 2143 (11 strains) of the 23S rRNA gene. At position 2142, 41 strains showed an A to G transition. The remaining 2 strains were:

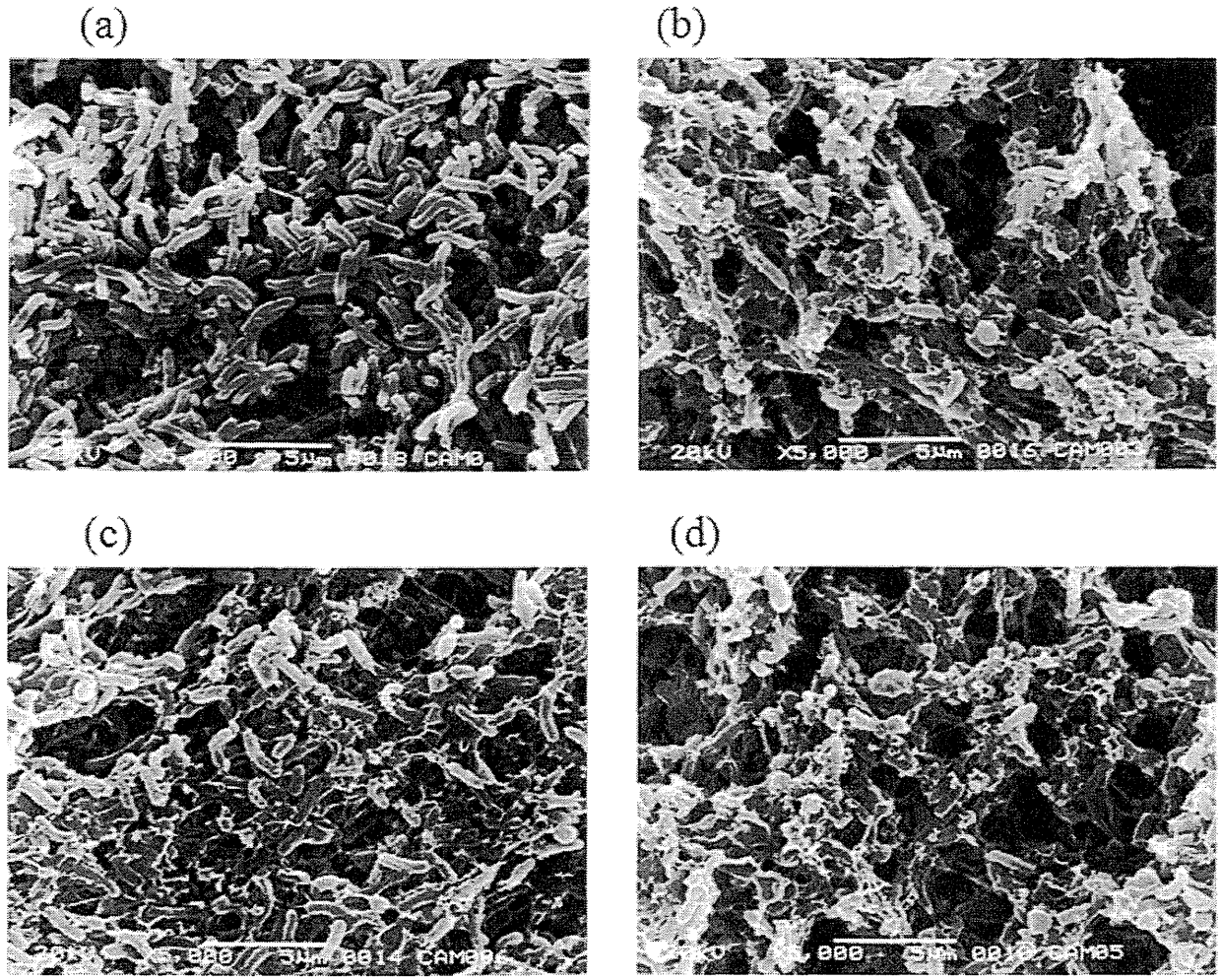

Figure 4. SEM images of TK1402 biofilm after various concentrations of CLR treatment. (a): the control cells (without treatment of CLR). (b): treated with 0.03 μg/ml of CLR. (c): treated with 0.06 μg/ml of CLR. (d): treated with 0.5 μg/ml of CLR. After treatment with the indicated concentrations of CLR, the biofilms were investigated using SEM. Scale bars are shown at the bottom of each electron microscope image.
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a strain from a 2-day biofilms after the third exposure to 0.5 μg/ml CLR exhibiting an A to T transition and a 2 day planktonic culture after the third exposure to 0.125 μg/ml CLR exhibiting an A to C transition. In the mutations at position 2143, all strains showed an A to G transition.

Discussion

In this study, we determined that biofilm formation in *H. pylori* increased the resistance to CLR at MIC levels by up to 4-fold in 2-day biofilms and to 16-fold in 3-day biofilms as well as MBC levels by up to 4-fold compared to planktonic cells (Fig. 1, Fig. 2, and Fig. 3). Similar phenomena of increased resistance to antibacterial agents have been reported in other biofilm forming bacteria [2,32,33]. In other bacterial species, multiple mechanisms of biofilm resistance to antimicrobial compounds were suggested (i) failure of the antimicrobial compounds to penetrate the biofilm, (ii) slow growth of the biofilm cells owing to nutrient limitation, (iii) activation of the general stress response [32,33,34,35,36,37]. Our present data showed that an increase in the biofilm biomass was observed after treatment with CLR (Fig. 2). However, the viability of the CLR treated biofilm cells was reduced in a dose dependent manner (Fig. 3). We hypothesize that these observations might reflect the time needed for CLR to diffuse because of the presence of the biofilm extracellular matrix (equivalent to (i) mentioned above). We previously demonstrated that the OMV produced by *H. pylori* strain TK1402 plays an important role in the formation of the extracellular matrix of the biofilm [10]. In addition, several studies indicated that the presence of extracellular DNA and mannose–related proteoglycans can contribute to the formation of biofilms as extracellular matrix components [38,39]. The extracellular matrix may exhibit a sequestering effect on CLR relative to internal cells within the biofilm. As a result, the biofilm biomass is increased after treatment with CLR but with time CLR diffuses to the interior of the biofilm followed by a decrease in cell viability. However, little is currently known regarding biofilm resistance in this microorganism and other mechanisms may also contribute to resistance. Specifically, participation of the efflux pumps of the RND family concerned with the development of antibiotic resistance has been well studied in *H. pylori* [26,30,31]. We

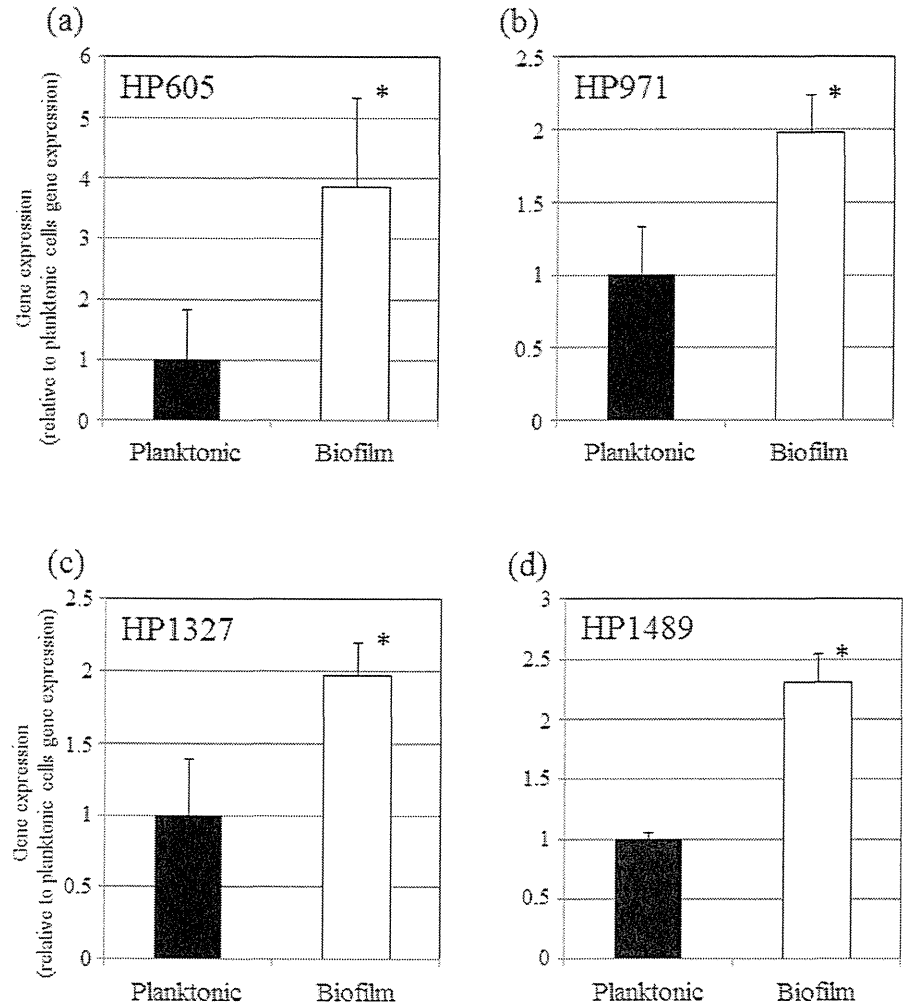

Figure 5. Expression of *H. pylori* efflux pump genes, HP605 (a), HP971 (b), HP1327 (c) and HP1489 (d). The quantity of cDNA corresponding to these genes was determined by real-time PCR and was normalized to that of the 16S rRNA gene in each unique reaction. Each experiment was repeated three times with at least duplicate samples from each independently isolated RNA preparation. Data are expressed as the means of all of experiments ± standard deviations. *significantly different ($p<0.05$) relative to the mRNA expression level (planktonic versus biofilm).
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analyzed the expression of mRNA for the efflux pumps genes (HP605, HP971, HP1327, or HP1489), and the expression of these genes was significantly more elevated in the biofilm cells than in the planktonic cells (Fig. 5). These results suggested that the high level of these genes transcript could contribute to biofilm resistance to CLR. To further test the potential contributions of other mechanisms, we analyzed the susceptibility of planktonic cultures at early exponential phase and stationary phase to CLR using a culture method and the late stationary phase cells were more resistant at 0.06 μg/ml than early exponential phase cells (data not shown). This result suggested indirectly that the slow growth of *H. pylori* cells might reduce the antimicrobial activity of CLR. Taken together, these observations suggested that there are multiple resistance mechanisms that could account for *H. pylori* biofilm cell resistance to CLR. Further characterization will be required to delineate the resistance mechanisms of biofilm cells.

In the previous *H. pylori* whole genome analysis, two copies of the 23S rRNA gene were detected in this microorganism [40,41,42]. We determined the properties of the two copies of the 23S rRNA gene in the strain TK1402 chromosome using Southern blotting (data not shown). When we examined the mutation sites of 23S rRNA by sequencing analysis, only one nucleotide was identified at positions 2142 or 2143 in the 23S rRNA of all samples. If only one copy of the 23S rRNA gene was mutated, equal amounts of PCR products from the mutated and wild-type copies should be amplified, indicating that both copies of the 23S rRNA at position 2142 or 2143 were mutated in all CLR resistant strains generated in this study. Taylor et al. reported that the majority of CLR resistant *H. pylori* require mutations in both copies of the 23S rRNA gene to confer CLR resistance [40], and this is consistent with our sequencing results.

Previous reports have indicated that mutations related to CLR resistance are generated at a very low frequency during *in vitro* CLR passage [43,44]. On the other hand, it is obvious that CLR resistance mutations were frequently generated in our present study, especially during exposure to 0.25 μg/ml of CLR, where the rate was 75% and 85% in 2-day and 3-day biofilms, respectively (Fig. 6b and 6d). The highly effective generation of

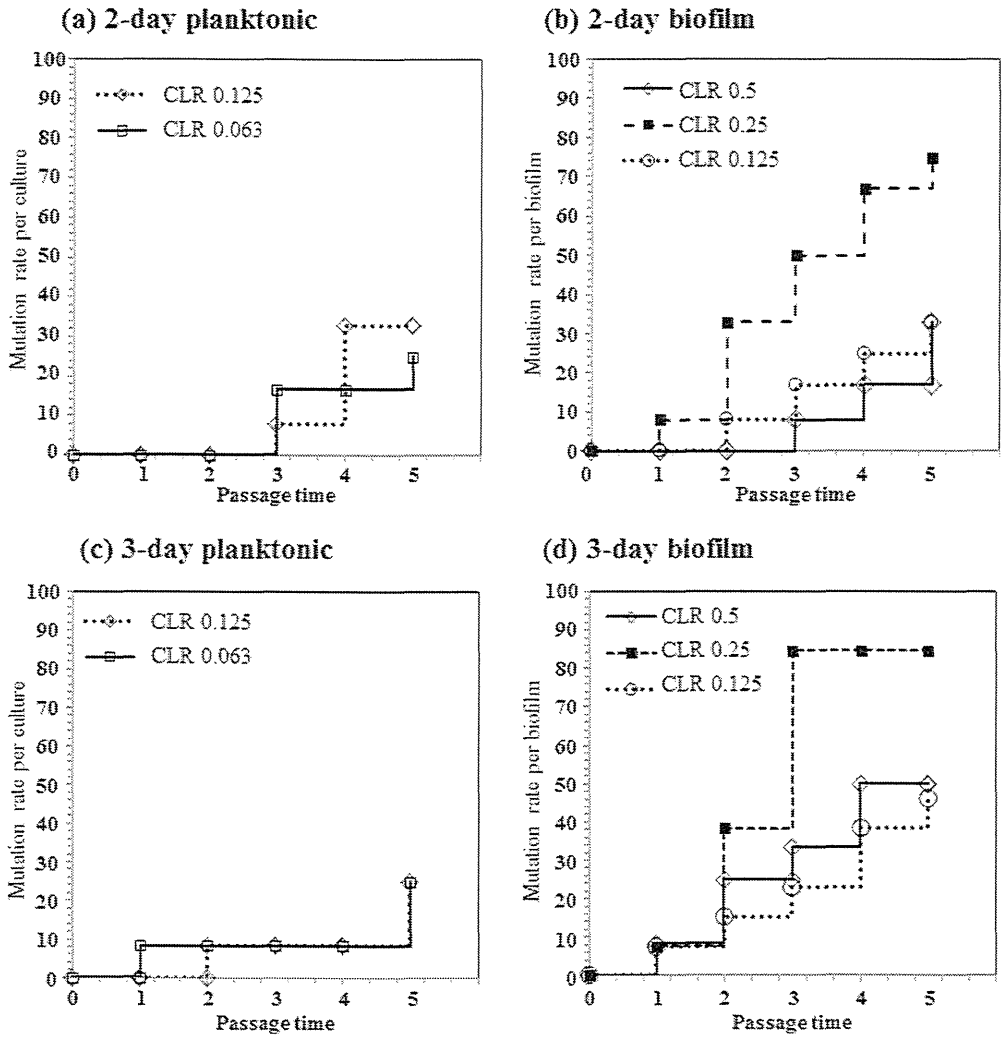

Figure 6. Induction of CLR resistance mutations in biofilm and planktonic cells. 2-day and 3-day biofilms (A and B) and planktonic cells (C and D) were exposed to each concentration of CLR. After an additional incubation for 24 h, cells were recovered in fresh Brucella-FCS agar, and the generation of CLR resistant mutants was assessed Brucella-FCS agar supplemented with 1.0 μg/ml CLR. When no CLR resistant cells were detected, exposure to CLR was repeated up to 5-times. The graph shows the accumulation ratio of the generated CLR resistant biofilm or planktonic cultures.
doi:10.1371/journal.pone.0073301.g006

CLR resistant mutants may be related to the concentration of CLR used in addition to the formation of biofilms. CLR concentrations used in previous reports were around one-half of the MIC but we were able to use one-eighth to one-half the MBC concentration of CLR (equivalent to 8- to 32-fold the MIC of strain TK1402). This relatively high concentration, especially one-quarter of MBC (0.25 μg/ml) of CLR may facilitate the generation of CLR resistance mutations. Further research is now in progress to examine this possibility.

CLR is well distributed throughout the human body and achieves high concentrations in tissue. Nakamura et al. reported that CLR concentrations in gastric juices, mucosa or serum after administration of 500 mg of the drug for 7 days were 550.6, 64.6 or 2.5 μg/ml at 2 hours after administration, and 43.4, 36.2 or 2.2 μg/ml at 6 hours, respectively [45]. These concentrations were sufficient to reduce the levels of *H. pylori* in vivo so that this microorganism formed biofilms. However, to reach such high concentrations of CLR in gastric mucosa for extended periods, the drug needs to be taken with sufficient dosage. In addition, in cases with inadequate compliance with eradication therapy, the concentration of CLR does not reach high concentrations in the gastric mucosa. Further, macrolides including CLR are frequently used in the treatment of various infectious diseases in pediatric, respiratory and otorhinolaryngology settings. In these cases, biofilm formation by *H. pylori* may contribute to the acquisition of CLR resistance. There are few studies in the literature regarding the relevance of mutational events in biofilm antibiotic resistance [46,47]. To our knowledge, this is the first demonstration that biofilm formation can affect the generation of antibiotic resistance mutations in *H. pylori*.

In some countries including Japan, triple therapy containing CLR is the best option for eradication of *H. pylori*. CLR resistance in *H. pylori* has serious implications for first-line eradication therapy in such countries, since it is thought to be the major factor in eradication failure, although other antibiotics such as amoxicillin were prescribed [15]. Our present study demonstrated that

the biofilm forming ability of *H. pylori* contributes to the development of CLR resistance and increases the frequency of the development of CLR resistant mutants relative to planktonic cells. However, since susceptibility to antibiotics has traditionally been evaluated using planktonic cells, and previous studies have shown that *H. pylori* forms biofilm on human gastric mucosa [7,8,9], these MICs are not reliable predictors of the antibiotic effects in the human stomach. CLR resistance in *H. pylori* can therefore be acquired by the selection of spontaneous mutation events that occur due to the magnitude and duration of macrolide use on the human gastric mucosa. Recently, a clinical trial for effective strategies targeting *H. pylori* biofilm infection through the use of molecules such as *N*-acetylcysteine was reported, and the eradication rate was increased compared to that of a non-treated group [48]. This study and our present results suggest that the assessment of the ability to form biofilms in *H. pylori* could play an important role in preventing and controlling the generation of antibiotic resistance.

Author Contributions

Conceived and designed the experiments: HY TO S. Kamiya. Performed the experiments: HY. Analyzed the data: HY TO. Contributed reagents/materials/analysis tools: HY TO TH S. Kurata S. Kamiya. Wrote the paper: HY TO KO S. Kamiya.

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Multilocus sequence typing of DNA from faecal specimens for the analysis of intra-familial transmission of *Helicobacter pylori*

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This study used multilocus sequence typing (MLST) of total DNA extracted from faecal specimens to genotype *Helicobacter pylori* to analyse intra-familial transmission. Faecal DNA was extracted and amplified by nested PCR. The products were analysed by direct sequencing and the allele type was determined using an MLST website. Mother-to-child transmission was suspected in at least two of three families, and father-to-child transmission was suspected in one family.

INTRODUCTION

Infection by the Gram-negative microaerophilic rod *Helicobacter pylori* is associated with the development of chronic gastritis, peptic ulcers and gastric adenocarcinoma in humans (Kusters *et al.*, 2006). It is thought that one of the modes of transmission of *H. pylori* is between family members, and therefore the presence of infected family members is an important risk factor in children (Konno *et al.*, 2008). However, there have been few studies that have proven intra-familial infection using isolated family strains (Konno *et al.*, 2005; Nahar *et al.*, 2009). It is difficult to isolate *H. pylori* from the gastric mucosa of children, as endoscopic analysis is not often undertaken. Therefore, the genotypes of paediatric *H. pylori* strains have not been analysed fully in comparison with those of other adult family members.

Multilocus sequence typing (MLST) analysis has become the most common method for genetic analysis of bacterial strains. MLST has been applied previously for the root causal analysis of outbreaks (Chalmers *et al.*, 2008), hospital infections (Walker *et al.*, 2012) and intra-familial infections (Staples *et al.*, 2012). An *H. pylori* MLST database is available online, consisting of seven housekeeping genes (http://pubmlst.org/helicobacter/), with over 2000 alleles detected at each locus. The genetic identity of various pathogenic strains can be analysed by MLST, which is tied in with information on the geographical sources of *H. pylori*, exposing major events in the history of human settlement (Achtman *et al.*, 1999; Falush *et al.*, 2001, 2003; Linz *et al.*, 2007; Moodley *et al.*, 2009; Wirth *et al.*, 2004).

After *H. pylori* organisms reach the anaerobic environment of the intestine, the micro-organisms are unable to grow and change morphology to their coccoid forms, which are non-culturable (Shirai *et al.*, 2000). However, the DNA of *H. pylori* has been reported to be detected by PCR using faecal specimens from infected patients (Scaletsky *et al.*, 2011) and animals (Oshio *et al.*, 2009). Here, we compared the MLST of faecal DNA specimens for the detection of intra-familial *H. pylori* infection.

Abbreviations: MLST, multilocus sequence typing; ST, sequence type.

METHODS

Participants. Children aged 0–12 years attending seven elementary schools, three nursery schools and six kindergartens in Sasayama city, Hyogo, Japan, were recruited into an epidemiological study. The Sasayama Study, for *H. pylori* infection in children, was carried out from November 2010 to March 2011. Stool samples were collected from 783 children, and 15 samples gave positive results. Family members of the 15 stool antigen-positive children were asked to provide stool samples, and 35 people belonging to 12 families provided samples.

The Sasayama Study was undertaken in accordance with the Declaration of Helsinki with approval from the Ethics Committees of Kyorin University, Tokyo; Hyogo College of Medicine, Hyogo; and Aichi Medical University School of Medicine, Aichi. Informed consent was obtained from the parents of children and from participants.

Stool antigen test. The collected stool specimens were kept at −80 °C until use. A TestMate Pylori Antigen enzyme immunoassay

(Wakamoto) was used for selection of *H. pylori*-positive faeces according to the manufacturer's guidelines. Briefly, 30 mg faecal specimen was diluted with 1 ml diluent. Faecal solution (50 µl) was added to each well and mixed with the reagent. Absorbance at 450 nm/630 nm was measured using a spectrophotometer, and the cut-off value of the test was taken as 0.100.

DNA extraction. Total DNA of *H. pylori* antigen-positive faeces was extracted using a QIAamp Stool kit (Qiagen) according to the manufacturer's instructions. Briefly, 200 mg frozen faeces was used for each extraction, and 200 µl DNA solution in Buffer AE (Qiagen) was eluted at the final step. *H. pylori* 16S rRNA gene-targeted primers were used for detection of *H. pylori* DNA by real-time PCR (Osaki *et al.*, 2006) and confirmed the *H. pylori* antigen-positive faecal samples.

MLST. The primers used for MLST are shown in Table 1. Gene fragments containing the *efp*, *mutY*, *ppa* and *trpC* genes were amplified from *H. pylori*-positive specimens by nested PCR. For the first reaction, 10 µl 2× Ampdirect Plus buffer (Shimadzu), 0.1 µl BIOTAQ Hot Start DNA Polymerase (Bioline), 2 µl primer mix (10 pmol μl^{-1} each), 6.9 µl distilled water and 1 µl DNA sample made a reaction volume of 20 µl. The Ampdirect Plus buffer neutralizes inhibitory substances in biological samples, and, as a result, increases PCR detection. The reaction mixture was incubated in a TP600 thermal cycler (Takara). Ex-*Taq* (Takara) was used in the second PCR. The amplification program consisted of one cycle at 94 °C for 10 min (first PCR) or 1 min (second PCR) and followed by 40 cycles of 94 °C for 20 s, 50–58 °C for 45 s and 72 °C for 45 s, with a final cycle at 72 °C for 7 min. The PCR products were separated using a 2 % agarose gel, stained by ethidium bromide and visualized under UV light. If two bands were visualized on the gel, only the band identical in size to the control band was collected.

The products were analysed by direct sequencing. Sequencing reactions were performed in a Bio-Rad DNA Engine Dyad PTC-220 Peltier thermal cycler using an ABI BigDye Terminator v3.1 Cycle Sequencing kit with AmpliTaq DNA polymerase (FS Enzyme; Applied Biosystems), according to the protocol supplied by the manufacturer. Single-pass sequencing was performed on each template using one of the second-PCR primers (forward or reverse, Table 1). The fluorescently labelled fragments were purified from the unincorporated terminator nucleotides by ethanol precipitation. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The direct sequencing results obtained were submitted to the MLST website, and the closest allele typing of each gene was determined.

Using the allelic profile of the four genes, the sequence type (ST) of each faecal sample was also determined through the MLST website by the nearest match.

RESULTS AND DISCUSSION

Fifteen stool antigen-positive children were found in the Sasayama Study. Family members of the 15 children were asked to provide stool samples, and 35 people from 12 families provided samples. We selected three families for MLST analysis according to the following; the proband child was diagnosed with *H. pylori* twice by a positive stool antigen test at 0 and 3 months, and the child had two stool antigen-positive family members. The remaining nine families did not match these conditions. All stool antigen-positive faeces were also positive for *H. pylori* DNA by PCR with no false-positive results.

MLST profiles of the *H. pylori* DNA extracted from faeces were determined in the three families in which there was an *H. pylori*-positive child and two family members (Table 2). In family A, *H. pylori* DNA and antigens were detected from the child and the parents but not from the sibling. The first faecal DNA sample of child A had four genes identical to its father. The second sample had the same *mutY* allele as its parents and the same *efp* allele as its mother.

In families B and C, *H. pylori* DNA and antigens were detected in the children, mothers and grandfathers but not

Table 1. Primers used in this study

Locus	PCR	Name	Primer*	Amplicon (bp)	Reference
efp	First	efp_for1	GGCAATTTGGATGAGCGAGCTC	558	MLST website
		efp_rev1	CTTCACCTTTTCAAGATACTC		MLST website
	Second	efp_for2	GGGCTTGAAAATTGAATTGGGCGG	500	MLST website
		efp_rev2	GTATTGACTTTAATGATCTCACCC		MLST website
mutY	First	mutY_for4	TTATGAAGTCTCTATATCAGCGAAGT	529	This study
		mutY_rev 4	TACCTAAACAATAAGGATTGAAAGG		This study
	Second	mutY_for 5	ATATCAGYGAAGTGATGAGC	516	This study
		mutY_rev 5	CCYAAACAATAAGGRTTKGAA		This study
ppa	First	ppa_for1-1	GAARTKAGCCATGACGCTRA	698	MLST website
		ppa_rev 4	GGGTTAARATCGTTAAATTGTAG		MLST website
	Second	ppa_for 1-2	AGCCATGACGCTRAKYCTTT	490	This study
		ppa_rev 1-2	CTCTTTGTTTTCAAACCCCTTG		This study
trpC	First	trpC_for8	AGCATCGCCCTCTAAAGGTT	618	This study
		trpC_rev 6	AAGCCCGCACACTTTATTTTC		This study
	Second	trpC_for 9	TCGCCCTCYAAAGGTTTRAT	564	This study
		trpC_rev 9	TCAAATCCTTTTCTTTCATYA		This study

*Y=C or T; K=G or T; R=A or G.

Table 2. MLST of faecal DNA in three families

Family	Family member*	Allele type for:			
		efp	*mutY*	*ppa*	*trpC*
A	Index child (1st)	1908†	703	1934	454
	Index child (2nd)	181	703	838	181
	Father	1908†	703	1934	454
	Mother	181	703	945	ND
	Sibling‡	–	–	–	–
B	Index child (1st)	1807	1540	502	1468
	Index child (2nd)	1807	1540	502	457
	Mother	1908	1540	502	1468
	Grandfather	1908	703	945	181
	Father‡	–	–	–	–
	Sibling‡	–	–	–	–
C	Index child (1st)	1908	2019	938	457
	Index child (2nd)	1908	703	1934	457
	Mother	1908	703	1934	1239
	Grandfather‡	–	–	–	–
	Father‡	–	–	–	–
	Sibling‡	–	–	–	–

ND, Not determined.

*1st and 2nd indicate the first and second samples taken, with an interval of 3 months between the first sample collection and the second.

†There were three differences from the 1908 allele sequence.

‡These family members were *H. pylori* negative.

from the faeces of siblings and fathers. The first faecal DNA sample from child B had identical allele types for the *mutY*, *ppa* and *trpC* loci but a different allele for the *efp* locus compared with those of the mother. The second sample from child B had identical *mutY* and *ppa* genes but different *trpC* and *efp* genes compared with its mother. The first faecal DNA sample from child C had the same *efp* gene only as its mother, but the second sample had identical alleles for *efp*, *mutY* and *ppa*.

The candidates for *H. pylori* sequence typing were defined from the database by combinations of MLST loci (Table 3). According to these data, in family A, the first sample of the child was shown to be same as that of its father and the second sample to be the same as its mother. In families B and C, the two isolates from the children were identified to be the same as those of their mothers.

We determined the source from whom the original strain was transmitted to the child. The implication was therefore that *H. pylori* was transmitted from mother to child in families B and C. In family A, *H. pylori* may have been transmitted either from the father and/or the mother. The results also implied that *H. pylori* strains from the grandfather were probably not the source of infection. In the Sasayama Study (from 2010 to 2011), no siblings of *H. pylori*-positive children were positive for faecal *H. pylori* antigen or the 16S rRNA gene. In this study, the infection rate was also very low. This may show that intra-familial transmission of *H. pylori* is rare.

Seven loci (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, *vacA* and *yphC*) of housekeeping genes are available for MLST analysis of *H. pylori* isolates. These are widely used markers for genomic diversity within *H. pylori* populations (Yamaoka, 2009). For the MLST analysis using faecal specimens, we used PCR to examine the above seven loci. It was difficult to obtain amplification products of *H. pylori* DNA >600 bp from faecal DNA due to the presence of either other bacterial DNA or substances inhibitory for PCR amplification. In addition, it may have been that the levels of *H. pylori* DNA were relatively low in the gut or that the DNA was damaged.

For the identification of allele types of *atpA*, *ureI* and *yphC*, fragments of ~600 bp (actually sizes of 627 bp, 535–585 bp and 504–631 bp, respectively) were needed to be amplified using available primers, so we instead selected the four shorter-length fragments (*efp*, *mutY*, *ppa* and *trpC*) for MLST using faecal samples.

In another study, we also showed that in one family the MLST profile of the child's *H. pylori* isolate from gastric mucus was identical to that of his mother's strain but not to that of the father's strain (data not shown). *H. pylori* MLST may therefore be useful as a tool for detection of the source of intra-familial infection.

Mother-to-child transmission occurs in early childhood and has been thought to be the most probable route of transmission of *H. pylori* in various countries including Bangladesh (Nahar *et al.*, 2009) and Japan (Konno *et al.*, 2008). In our study, mother-to-child transmission was suspected in two or three of the three cases analysed, whilst father-to-child transmission was suspected in one case. Furthermore, grandparent-to-child transmission was not detected. Our study indicated that parents can be a potential source of *H. pylori* infection in children.

The alleles of *trpC* belonged to different ST types in family C, and the *ppa* allele was different in two samples. One possible explanation is that multiple strains with different alleles had colonized the child or, less likely, that these genes had mutated in the 3-month study period.

It was reported by Raymond *et al.* (2004) that analysis of the isolates from family members indicated natural mixed infection in the family. Identical alleles were found in some strains isolated from the children and parents, demonstrating that strains had circulated within the family.

It is well known that high genetic diversity is a hallmark of *H. pylori*. Kennemann *et al.* (2011) reported very few mutations in an isolate cultured for 3 months after infection of a human volunteer, highlighting the importance of mixed infections for genetic diversification of *H. pylori* through recombination. As it has been reported that *H. pylori* strains exhibit diverse genotypes after long-term infection from childhood to adulthood (Kraft, *et al.*, 2006),

Table 3. Results of MLST

Family	Member*	Candidates for MLST (STs)†	Family member with similar STs
A	Child (1st)	**960/1660/2250/2265**	Father
	Child (2nd)	**181**	Mother
	Father	**960/1660/2250/2265**	
	Mother	**181**/664/960/975/978/1143/1145/1262/1264/1403/1445/1733	
B	Child (1st)	**489/1108/1346/1466/1565/1929**/2145	Mother
	Child (2nd)	**489**/669/**1108**/1290/**1466/1929**/2145	Mother
	Mother	**489/1108/1346/1466/1565/1929**/2265	
	Grandfather	181/960/1228/2265	
C	Child (1st)	669/870/1290/**2250/2265**	Mother
	Child (2nd)	669/1290/2207/**2265**	Mother
	Mother	960/1809/**2250/2265**	
	Grandfather	402/2269	

*1st and 2nd indicate the first and second samples taken, with an interval of 3 months between the first sample collection and the second.
†STs that were the same in each family are indicated in bold.

it is likely that intra-familial transmission of *H. pylori* can be determined by a molecular technique such as MLST.

In conclusion, these results demonstrated that MLST of faecal *H. pylori* DNA is a useful tool for the detection of intra-familial transmission.

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