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# An integrated microfluidic system for diagnosis of the resistance of *Helicobacter pylori* to quinolone-based antibiotics ☆

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ABSTRACT

*Helicobacter pylori* (*H. pylori*) is a species of bacteria that can colonize the human stomach mucosa. It is closely associated with gastric diseases such as ulcer and inflammation. Recently, some *H. pylori* strains were found to express resistance to a family of antibiotics known as quinolones due to single-point mutations. Although traditional polymerase chain reaction (PCR) and molecular diagnostic-based approaches can be used to determine the presence and abundance of antibiotic-resistant *H. pylori* strains, such processes are relatively expensive, labor-intensive, and require bulky and costly equipment. This study therefore reports an advanced diagnostic assay performed on an integrated microfluidic system for rapid detection of antibiotic resistance in *H. pylori*. The assay features three components: (1) nucleic acid extraction by specific probe-conjugated magnetic beads, (2) amplification of the target deoxyribonucleic acid (DNA) fragments by using single-nucleotide-polymorphism polymerase chain reaction (SNP-PCR), and (3) optical detection of the PCR products. The device integrates several microfluidic components including micro-pumps, normally-closed micro-valves, and reaction chambers such that the entire diagnostic assay can be automatically executed on a single microfluidic system within one hour with detection limits of $10^0$, $10^2$, and $10^2$ bacterial cells for *H. pylori* detection and two different SNP sites strains. Three PCR-based assays for determining presence of *H. pylori* infection and two DNA single-point mutation assays aimed at determining whether the infected strains were resistant to quinolone can be performed simultaneously on a single chip, suggesting that this microfluidic system could be a promising tool for rapid diagnosis of the presence of antibiotic-resistant *H. pylori* strains.



## 1. Introduction

*Helicobacter pylori* (*H. pylori*) is a spiral-shaped, Gram-negative microaerophilic bacterium that colonizes the human stomach mucosa (Marshall and Warren, 1984). About 90–100% of duodenal ulcer and 60–100% of gastric ulcer patients were also characterized by *H. pylori* infection (Kuipers et al., 1995). *H. pylori* might also lead to gastric cancer and primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue (MALT) (Uemura et al., 2001; Wotherspoon et al., 1993). Early diagnosis and treatment could enhance patients' well-being and decrease the ultimate risk of gastric cancer; hence, early diagnosis of *H. pylori* infection is of critical importance. Several approaches are currently employed to confirm *H. pylori* infection, and these include symptom-based diagnosis and endoscopy alongside biopsy sampling (Makris et al., 2003; Cohen and Laine, 1997). Such biopsies require histological analysis, and the bacterial communities must be cultured, which is a relatively time-consuming and labor-intensive process.

Recently, a treatment known as “triple therapy” has been commonly adopted to eradicate *H. pylori* (Logan et al., 1991). This therapy consists of one proton pump inhibitor and two different antibiotics. The function of the proton pump inhibitor is to enhance the efficacy of the antibiotics by controlling the pH of the stomach. However, this treatment is not 100% successful, as some *H. pylori* strains have evolved to be resistant to commonly used antibiotics (Adamek et al., 1998). After one to two weeks of treatment, if the symptoms of infection still exist, a second biopsy check is recommended, and the treatment period is prolonged;

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this means that the patient will bear the discomfort longer, and moreover, experience elevated medical costs. As such, accurate detection of *H. pylori* strains demonstrating drug resistance in patients' biopsies is essential.

Regarding antibiotic resistance, some other antibiotics have been considered as part of a secondary eradication treatment regimen for *H. pylori*. In recent reports, a 7-day regimen of gatifloxacin (8-methoxy fluoroquinolone), amoxicillin, and rabeprazole was investigated to be an effective and safe treatment combination (Nishizawa et al., 2009). The quinolones are in the nalidixic acid family. Deoxyribonucleic acid (DNA) gyrase plays an important role in DNA duplication by relieving the tension resulting from the unwinding of DNA. Quinolones target bacterial DNA gyrase such that their replication and hence growth is thwarted. Recently, some DNA point mutations have been reported to cause resistance to quinolones in *H. pylori* (Umegaki et al., 2000). Two mutation sites resulting in single amino acid substitutions were found to lead to quinolone-resistance frequently are located at the 87th amino acid (asparagine to lysine shift; the abbreviation of the mutation site is N87K) and the 91st amino acid (aspartic acid to glycine shift; the abbreviation of the mutation site is D91N) of the gyrase A subunit (Hung et al., 2008). Therefore, the detection of these two single-point mutation is of great need for *H. pylori* treatment.

The epsilometer test and Kirby–Bauer disk diffusion susceptibility test are two routine methods to confirm the resistance to antibiotics (Bauer et al., 1966; Glupczynski et al., 1991). Both methods require a time-consuming *H. pylori* culture step. Moreover, as the cultured samples are usually taken from biopsies, problems like the biopsies getting contaminated on the way of transportation or the bacterial number in the biopsies being too low to culture successfully may occur. These unexpected situations might affect the test results. Alternatively, the polymerase chain reaction (PCR) is a molecular diagnostic technology that was developed to amplify target DNA with a pair of designed primers and several temperature cycles to solve the problem of the false negative result produced by the low number of bacteria (Yamamoto, 2002). As DNA is relatively stable, even if the bacteria die during transport of the biopsy, the method can still detect the presence of bacteria. Previous reports detailed that the 16S ribosomal ribonucleic acid (rRNA) DNA segment in *H. pylori*'s genomic DNA is a conserved gene of *H. pylori* (Ho et al., 1991). Some complementary 16S rRNA DNA PCR primers were therefore designed to confirm the presence of *H. pylori* in the sample (Mapstone et al., 1993). Furthermore, in order to detect the aforementioned mutation sites, the PCR amplicons are sequenced afterwards, thereby confirming whether or not *H. pylori* in the sample were of the antibiotic-resistant strain (Akopyanz et al., 1992; Occhialini et al., 1997). The method is precise; however, DNA sequencing has a relatively high cost and is relatively time-consuming. The entire process may take three hours or even longer, and most sequencing cores requires 1–2 days to return the sequence data. Moreover, the DNA sequencing machine itself is relatively expensive and is beyond the cost limits of most laboratories. Therefore, a cost-effective and fast diagnostic assay for *H. pylori* and its single-point mutation sites (N87K and D91N) is of great needs.

Recently, a technology called single-nucleotide-polymorphism polymerase chain reaction (SNP-PCR) has emerged, and this method, which is dependent upon allele-specific primer design, has been adapted to detect the single-point mutations that lead to quinolone resistance in *H. pylori* (Nishizawa et al., 2007; Nakamura et al., 2007). Briefly, an allele-specific primer has been designed to amplify the mutated DNA segment only and not the wild-type DNA. This method leads to an accurate diagnosis of antibiotic resistance, though it should only be performed by well-trained professionals. Some expensive and bulky apparatus such as PCR machines are also required. Alternatively, recent advances in micro-electro-mechanical-systems (MEMS) technology have improved the development of novel and compact experimental platforms. In the biosensor field, various compact chips have been designed to adopt different detection processes at micro-scale levels, and this technology is known as lab-on-chip (LOC) or micro-total-analysis-systems (μ-TAS) (Maluf, 2002; Reyes et al., 2002). For instance, microfluidic systems that integrate several modules on a single chip for performing DNA purification, collection, and nucleic acid amplification have been reported (Wang et al., 2013; Pereira et al., 2010; Chang et al., 2013b). The advantages of these novel experimental platforms include lower energy requirements, automated control systems within the micro-devices, and their ability to enable molecular diagnostics *in situ*. Furthermore, in order to decrease the presence of inhibitors in the samples and increase the target DNA collection efficiency, magnetic beads are commonly used to pre-treat samples (Hultman and Stahl, 1989; Levison et al., 1998). Specifically, nucleotide probe-conjugated magnetic beads were used to capture specific DNA fragments (Parham et al., 2007; Flagstad et al., 1999). In this study, an advanced microfluidic system which integrated micro-pumps, microvalves, has been reported to automate the entire diagnostic process for *H. pylori*. Furthermore, SYBR® Green I, a dye that fluoresces upon binding to double-stranded DNA, was incorporated in the PCR step to intercalate into the DNA fragments, and the consequent fluorescence intensity was measured directly in the developed microfluidic system. The study has performed SNP-PCR on an integrated microfluidic system, and the developed technology could allow for easy, accurate, rapid (within an hour), automatic detection of antibiotic resistant *H. pylori* strains.

The preliminary results have been published in the 27th IEEE International Conference on Micro Electro Mechanical Systems (Chao et al., 2014). The previously study reported that the results of the specificity and the sensitivity of the diagnosis assay. However, after more test, original *H. pylori*-specific primers produced some cross-reaction which causes unexpected PCR products with other bacteria. As for the diagnosis assay's sensitivity test, the samples we used to test previously were pure target DNA. It does not fit the need of clinical use. Therefore, to approve and confirm the specificity of the assay, a new pair of *H. pylori*-specific primers was redesigned and the diversity of tested bacteria increased. Besides, the samples used to examine the sensitivity of the assay were replaced from pure DNA to bacteria cells. Moreover, the multiple PCR were performed on a single chip. It went even further in clinical needs.

## 2. Materials and methods

### 2.1. Sample preparation

*H. pylori* (provided from National Cheng Kung University Hospital, Tainan, Taiwan) was collected from clinical samples and grown on Control and Disease Center (CDC) anaerobic blood agar (BBL, BD Inc., USA) at 37 °C under microaerophilic conditions and scraped off the plates. The bacteria were then suspended in 1 mL of phosphate buffered saline (PBS, pH 7.4). Concentrations of the bacterial suspensions were estimated by a spectrophotometer (Nanophotometer-pearl, Implen, Germany). According to previous studies, an optical density (OD, $\lambda = 600$ nm) 1.0 corresponds to $10^8$ colony forming units (CFU) mL of *H. pylori* (Deml et al., 2005; Simala-Grant et al., 2001). PBS was used as the diluting solution for preparing different bacterial numbers samples. *H. pylori* strains, including one reference strain (J99), two clinical single-point mutant N87K strains (1450 and 1520), and two clinical single-point mutant D91N strains (1444A and 1444B) were used in