



Quantitative detection for low levels of *Helicobacter pylori* infection in experimentally infected mice by real-time PCR

Michał Mikula^a, Artur Dzwonek^a, Katarzyna Jagusztyn-Krynicka^b, Jerzy Ostrowski^{a,*}

^aDepartment of Gastroenterology, Medical Center for Postgraduate Education, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, ul. Roentgena 5, 02-781 Warsaw, Poland

^bDepartment of Bacterial Genetics, Institute of Microbiology, Warsaw University, Poland

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Abstract

Accurate diagnosis of *Helicobacter pylori* infection is important in both clinical practice and clinical research. Molecular methods are highly specific and sensitive, and various PCR-based tests have been developed to detect *H. pylori* in gastric biopsy specimens. We optimized a sensitive and specific quantitative SYBR Green I real-time PCR assay for detection of *H. pylori* based on amplification of the fragment of a 26-kDa *Helicobacter* species-specific antigen gene that allows for detection of 5 bacterial cells per PCR sample. Under the assay conditions, SYBR Green I real-time PCR is highly reproducible with a precise log-linear relation in the range of six orders of magnitude of bacterial DNA concentrations. For accurate comparison of *H. pylori* infection in different tissue samples, the amount of total host DNA in each sample is normalized by TaqMan real-time PCR of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) pseudogenes. The developed method was validated in prophylactically immunized and experimentally infected mice and revealed a level of *H. pylori* gastric colonisation that was below the limit of detection for a rapid urease test. This new method established for a quantitative analysis of *H. pylori* in the host's stomach may be useful in experimental studies evaluating new anti-*H. pylori* drugs and vaccines.

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1. Introduction

Helicobacter pylori is a spiral, gram-negative bacterium that infects gastric mucosa with a prevalence rate of about 50%, ranging from 8% to 87% (Eurogast Study Group, 1993). It is considered to be the major causative agent of acute and chronic gastritis, and a

significant etiologic factor of peptic ulcer disease, gastric cancer, and MALT lymphoma (Blaser, 1990; Marshall, 1994; Cover and Blaser, 1995). Treatment of *H. pylori* involves expensive combinations of various medicines (antibiotics, proton pump inhibitors) and is not always effective. Hence, there is an urgent need to develop new approaches to management of *H. pylori* infection. Immunologically mediated protection against the infection poses an attractive alternative.

Accurate diagnosis of *H. pylori* infection is important in both clinical practice and clinical research,

* Corresponding author. Tel.: +48-22-6440102; fax: +48-22-6447601.

E-mail address: jostrow@warman.com.pl (J. Ostrowski).

and diagnostic tests available for the diagnosis of *H. pylori* infection include invasive and noninvasive methods (Leodolter and Megraud, 2001; Hirschl and Glupczynski, 1999; van Zwet and Megraud, 1998;Leodolter et al., 2001). All invasive, endoscopy-based tests are performed on biopsy specimens, and noninvasive tests can be done using serum, saliva, stool, or expired breath samples. The most accurate noninvasive test is the ^{13}C -urea breath test, but assays based on gastric mucosal specimens are most commonly employed in clinical practice. Of these, the simplest and most frequently used is the qualitative rapid urease test. Histological identification and culturing of *H. pylori* allow semiquantitative assessment of bacterial density at increased complexity and cost.

Molecular methods used as diagnostic tests are highly specific and sensitive, and various PCR-based tests have been developed to detect *H. pylori* in gastric biopsy specimens (Leodolter and Megraud, 2001; van Zwet and Megraud, 1998; Pacheco et al., 2001). However, in clinical practice simplicity and speed are required from diagnostic tests. Therefore, PCR techniques that remain too labour-intensive are not recommended for routine use in the diagnosis of *H. pylori* infection. On the other hand, these tests are ideal in experimental studies of experimental therapeutics and protective antigens for vaccination against *H. pylori* (Smith et al., 1997; Chisholm et al., 2001; Solnick et al., 2000).

For detection of PCR products different techniques are used, such as ethidium bromide, fluorescence or radioactive labeling after gel electrophoresis. However, for the quantification of PCR product it is extremely important to measure DNA amount during the exponential phase of reaction. Since real-time PCR measures the intensity of a fluorescent signal, which is proportional to the amount of amplification product, this method seems to be the most sensitive and accurate of all PCR quantification methods (Gulietti et al., 2001).

We describe a new sensitive and specific quantitative method for detection of *H. pylori* based on real-time PCR that employs the double-stranded DNA-binding dye SYBR Green I. The method was optimized and validated in experimentally infected mice for profiling low levels of *H. pylori* infection.

2. Materials and methods

2.1. Conventional PCR assay

PCR reactions were performed as described previously (Hennig et al., 1999). PCR amplifications were done in 25- μl $1 \times$ PCR reaction buffer containing genomic DNA, primers, 200 μM deoxynucleotide triphosphate (dNTPs), 1.5 mM MgCl_2 , and 1.0 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR reactions were run in a GeneAmp 2400 PCR System (Perkin-Elmer). PCR conditions were as follows: for amplification of the 26-kDa species-specific antigen (*SSA*) gene—10 min initial denaturation at 95 °C and 37 cycles of 30 s at 92 °C and 1 min at 68 °C; for amplifications of all other genes—10-min initial denaturation, and 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The last cycle had the final elongation increased to 7 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining.

2.2. Quantitative real-time PCR assay

Amplification, data acquisition, and data analysis were carried out using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). To amplify *H. pylori* DNA, the real-time PCR used double-stranded DNA-specific dye SYBR Green I. The reaction mixture contained template DNA, 12.5- μl $2 \times$ SYBR Green PCR Master Mix (Applied Biosystems), and 50 nM primers at a final volume of 25 μl . The reactions were cycled using the same parameters as in conventional PCR. Amplification of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) pseudogenes was performed using the TaqMan (Applied Biosystems) real-time PCR. The reaction mixture contained template DNA, 12.5- μl $2 \times$ PCR buffer (TaqMan[®] Universal PCR Master Mix), 100 nM TaqMan fluorescence-labeled probe, and 300 nM primers at a final volume of 25 μl . The reactions were cycled using the following parameters: 10-min initial denaturation at 95 °C and 35 cycles of 95 °C for 15 s and 60 °C for 1 min. All assays were carried out in 96-well format plates in triplicate. In all amplification reactions, template negative reactions were also included.

2.3. Animals

C57BL/6 mice were housed under specific pathogen-free condition and constant room temperature with a 12:12-h light–dark and permitted free access to water and to standard food pellets. The animals received humane care in compliance with the regulations of Cancer Center, and experiments were performed in accordance with the guidelines of the Polish Ethical Committee for Animal Experiments.

2.4. Bacterial strain

The *H. pylori* Sidney Strain (SS1), kindly provided by Dr. A. Lee (Lee et al., 1997), was grown on Blood Agar Base No. 2 medium containing an antibiotic mixture for 1–2 days at 37 °C under microaerobic conditions in GasPak jar with the CampyPak system. The preculture was used to inoculate liquid Tryptone Soya Broth medium supplemented with 5% fetal bovine serum and antibiotics. The flasks were kept under microaerobic conditions at 37 °C with constant shaking (150 rpm), and bacterial cells were used for inoculation and as source of *H. pylori* immunogens.

Chromosomal DNA from *H. pylori* SS1 was purified using Genomic DNA Prep Plus (A&A Biotechnology) according to manufacturer's instruction. Aliquots of bacterial DNA were used for the quantification by spectrophotometry.

2.5. *H. pylori* antigen preparation

To obtain *H. pylori* whole-cell lysate antigens, the bacterial cultures were centrifuged, the pellet was washed three times with PBS, the cells were sonicated, and after centrifugation the supernatant was collected and stored at –80 °C.

2.6. Expression and purification of recombinant urease B

Subfragment of urease B (*UreB*) was amplified from *H. pylori* genomic DNA by PCR using the following pair of primers: sense 5'-GGATCCA-CAGGCGATAAAGTGAGATT-3' containing *EcoRI* site (in italics) and antisense 5'-TTTCTTCTGCCTG-GAGTGAT-3'. The PCR product was subcloned into

pCR2.1-TOPO as per manufacturer's protocol (Invitrogen). Then the plasmid was digested with *Bam*HI and *Eco*RI and the gel-purified insert was subcloned into bacterial expression vector pGEX 4T-1 (Amersham Pharmacia Biotech), which was used to produce *rUreB* fused to the C terminus of glutathione-S-transferase (GST). The plasmid was transformed into *E. coli* BL21 DE3 pLysS cells (Novagen), and bacterial cells were grown until they reached OD=0.6 and then were treated for 3 h with 0.5 mM IPTG. Cells were harvested by centrifugation, and following freezing and thawing, the bacterial pellet was suspended in PBS containing 5 mM DTT, 0.1 mM leupeptin, 0.5 mM PMSF, and 0.1 mM lysozyme, and sonicated on ice. After centrifugation, recombinant GST-UreB was purified by affinity chromatography using glutathione-agarose column, and then was cleaved using thrombin protease (Amersham Pharmacia Biotech) according to manufacturer's protocol to obtain pure *rUreB*.

2.7. Vaccination and infection

Outbred specific pathogen-free C57BL/6 mice were immunized orally via feeding needles with *H. pylori rUreB* (50 µg p.o.) plus cholera toxin (5 µg p.o.) or with *H. pylori* whole-cell lysate antigens (50 µg p.o.) plus cholera toxin (5 µg p.o.) suspended in PBS. Vaccination was repeated at week intervals for 4 weeks with the same doses. Six weeks after the last vaccination, sham-immunized mice and those immunized with *H. pylori*-specific antigens were challenged two times at weekly intervals by gastric gavage with 4×10^8 of live *H. pylori* SS1 cells, suspended in 300 µl of 0.2 M NaHCO₃. Three weeks after the last challenge, animals were sacrificed and stomachs removed by aseptic technique. Then stomachs were cut longitudinally, gently washed with sterile water, and gastric mucosa was gently scraped with a sterile glass. Fragments of mucosa specimens were detected for *H. pylori* colonisation using the qualitative rapid urease test (CLO test) which was read routinely 1 h after gastric mucosa specimen collection and, if negative, 3 and 24 h later. Development of a pink colour was indicative of a positive test. Other portions of gastric mucosa were frozen at –80 °C until use for DNA preparation.

2.8. DNA preparation

DNA from gastric mucosa samples was extracted as previously described (Hennig et al., 1999). In brief, gastric mucosa was digested with proteinase K (0.5 µg/µl) in 50 mM Tris-HCl, pH=8.0, 1 mM EDTA, and 0.5% Tween 20 at 56 °C for 3 h. Tissue lysates were further purified on 5% Chelex 100 (Perkin-Elmer), and DNA was recovered by extraction with equal volume of phenol-chloroform and precipitation with ethanol. DNA pellets were dissolved in TE buffer, and after quantification in a spectrophotometer, DNA samples were stored at -80 °C.

3. Results and discussion

In this study we developed a method to detect and quantify low levels of *H. pylori* infection. As described below, we have defined the experimental conditions for real-time PCR using the amplification of the fragment of 26-kDa *Helicobacter* species-specific antigen (*SSA*) gene (O'Toole et al., 1991) that allowed for detection of 5 bacterial cells per PCR sample.

Real-time PCR measures a fluorescent signal that is proportional to the amount of amplified DNA. The most reliable point for quantification of template DNA is the cycle number at which the PCR product fluorescence becomes greater than a defined threshold. The fluorescence threshold represents the cycle number (Ct cycle) at which dye binding to PCR product generates a signal approximately 3 standard deviations above background. An arbitrary threshold level is inversely proportional to the log of the initial amount of template DNA. Thus, the more starting template DNA, the fewer PCR cycles are required to reach the threshold (Gulietti et al., 2001).

The fluorescence signal is produced by intercalating dyes such as SYBR Green I or by sequence-specific labeled probes (Gulietti et al., 2001; Wittwer et al., 1997). Although TaqMan sequence-specific fluorescence-labeled probes are very specific, they are relatively expensive. In contrast, SYBR Green I dye can be used with any pair of primers for amplification of any DNA and, therefore, it is a much more economical alternative for quantitative PCR.

3.1. SYBR Green I real-time PCR

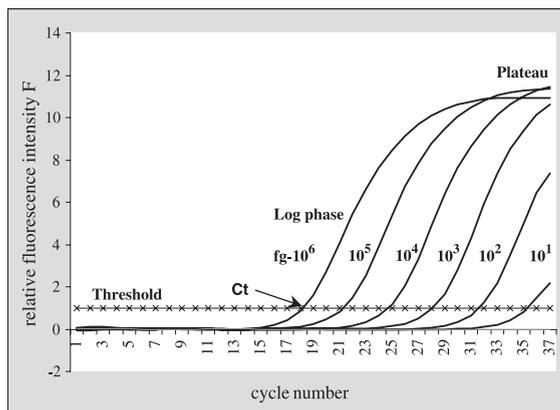
SYBR Green I can incorporate into any double-stranded DNA including unspecific products, such as primer-dimer. Since extensive accumulation of primer-dimer is often observed in PCR reactions with low amount of target DNA or in no-template control samples, SYBR Green I PCR can produce false-positive results. The goal of our study was to optimize and validate an SYBR Green I real-time PCR assay for profiling low level of *H. pylori* infection.

Different PCR amplicons and nonspecific amplification products can accurately be distinguished by generation of DNA melting curves (Al-Robaiy et al., 2001). Initial experiments were designed to select PCR reaction conditions that generated the lowest amount of unspecific PCR products. For this purpose, five pairs of primers targeting for *H. pylori* *UreB*, *SSA*, *Hp1117*, *Hp1042*, and *Hp1046* genes were used, and PCR conditions were determined empirically (not shown). The specificity of the PCR assay for each pair of primers was evaluated by adding 10-fold dilutions of purified *H. pylori* DNA to gastric mucosa DNA obtained from uninfected mice. Both conventional PCR that was evaluated by agarose gel electrophoresis and real-time PCR that was evaluated by plotting the melting curves and gel electrophoresis revealed the highest specificity of PCR reactions with primers designed for the *SSA* gene. These primer sequences were as follows: sense: 5'-TGGCGTGTCTATTGACAGCGAGC-3'; antisense: 5'-CCTGCTGGGCATACTTCACCATG-3'. In further studies on quantification of *H. pylori* infection level by real-time PCR, we amplified this fragment of the *SSA* gene.

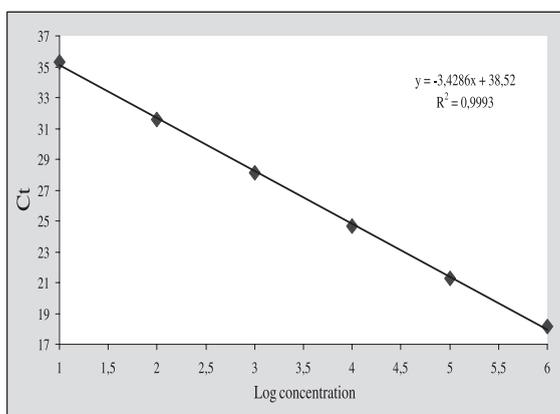
We first determined the sensitivity of the quantitative PCR assay for detection of the *H. pylori* *SSA* gene. Serial 10-fold dilutions of chromosomal DNA from pure *H. pylori* SS1 culture in a background of 200-ng mouse genomic DNA were used as the PCR templates. Fig. 1A shows a representative experiment on samples containing 10¹–10⁶ fg of bacterial DNA. The corresponding calibration curve was obtained by plotting the initial amount of bacterial DNA in the standard series against the corresponding fluorescence Ct cycle number (Fig. 1B). Correlation coefficients obtained by linear regression analysis of several independent experiments were higher than R²=0.99. Thus, under the assay conditions, SYBR Green I real-

time PCR was highly reproducible with a precise log-linear relation in the range covering at least 6 log of bacterial DNA concentrations.

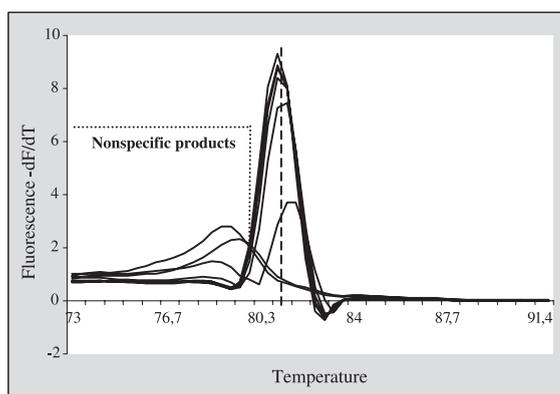
A.



B.



C.



To determine the specificity of real-time PCR reactions, a melting curve analysis was performed. As shown in Fig. 1C, in the samples containing 10^1 – 10^6 fg of bacterial DNA, a single product with a melting temperature of 81 °C was observed, and nonspecific amplification had no impact on the quantification of *H. pylori*. Dilutions containing less than 10 fg of *H. pylori* DNA and 200 ng of mouse genomic DNA gave only nonspecific amplification product.

The genome size of *H. pylori* is estimated as 1.65 Mb; since the molecular weight for 1 bp is 650, the bacterial genome is equal to approximately 1.07×10^9 g/mol and contains 6.02×10^{23} molecules/mol (Avogadro's number). Thus, 10 fg of chromosomal DNA is equivalent to about 5 *H. pylori* cells.

3.2. Taqman real-time PCR

For accurate comparison of *H. pylori* infection in different tissue samples, the amount of total host DNA in each sample requires normalization. Due to the known presence of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) retroprocessed pseudogenes in the mouse genome (Garcia-Meunier et al., 1993), the quantitative PCR of *GAPDH* was used for quantification of mouse genomic DNA per sample and to correct for the presence of compounds in the DNA sample that could potentially inhibit PCR reactions. The PCR reactions were performed using the TaqMan chemistry. TaqMan primers and probe were designed with PrimerExpress I Software (Applied Biosystems). The nucleotide sequences of primers and probe are given in Fig. 2. The probe was labeled with FAM (6-carboxyfluorescein, a fluorescent reporter) and with TAMARA (6-carboxytetramethylrhodamine, a fluo-

Fig. 1. (A) Logarithmic amplification plots from an assay of 26-kDa *Helicobacter* species-specific antigen (*SSA*) gene. Six 10-fold serial dilutions containing 10^1 – 10^6 fg of bacterial DNA were amplified in the presence of 200 ng of mouse genomic DNA by real-time PCR using SYBR Green I dye on a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). The software constructed amplification plots, where ΔR_n is plotted against cycle number. (B) Standard curve for *SSA*. C_T values were plotted against input template DNA. (C) Melting curve analysis for PCR end products of the *SSA* gene obtained after the completion of a real-time PCR run using SYBR Green I dye. The reduced melting temperature of the product formed in PCR with a low amount of target DNA or in no-template control samples is characteristic of primer-related nonspecific amplification (primer–dimer).

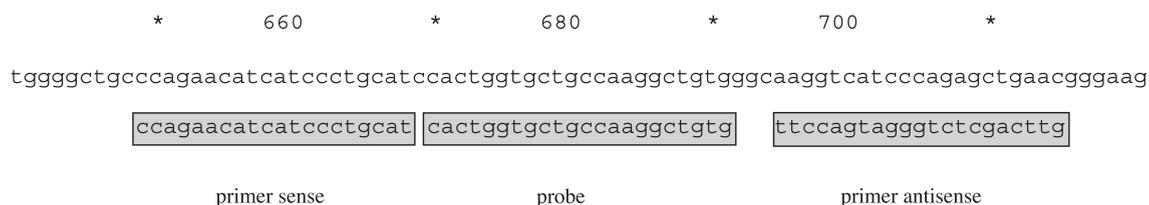


Fig. 2. Primers and probe used in TaqMan real-time PCR assay for *GAPDH* pseudogenes. Mouse *GAPDH* was derived from GenBank; accession number M32599.

rescent quencher). The minimal primer concentrations optimal for PCR were determined empirically accordingly to manufacturer's guidelines (data not shown).

Calibration curves were generated by analyzing dilutions of *GAPDH* DNA amplified by standard PCR, gel-purified, and measured in a spectrophotometer, as well as by testing 10-fold serial dilutions of known amounts of mouse genomic DNA. For this purpose, each calibrator was correlated with its threshold cycle value and then the calibration curve was generated (Fig. 3). The results of these experiments show that TaqMan amplification of *GAPDH* was highly reproducible over a wide dynamic range.

3.3. Quantification of mouse samples

Applicability of this new quantification assay was tested in gastric mucosa samples harvested from control (*Helicobacter*-free) mice and those experimentally infected that were previously sham-immunized or immunized with *H. pylori*-specific antigens. The number of bacterial cells and the amount of DNA present in each sample were quantified by interpolation of the corresponding Ct values in the standard curves for *H. pylori* chromosomal DNA and gastric mucosa *GAPDH*. Fig. 4 illustrates that 3 weeks after challenge, all sham-immunized mice were well colonized with *H. pylori*. The extrapolated amount of bacterial chromosome DNA quantified by real-time PCR ranged from 674 to 41.559 fg *H. pylori* DNA/100 ng mouse genomic DNA, which corresponded to 379–20779 bacterial cells (Fig. 4). In contrast, no bacterial DNA was found by real-time PCR in any gastric sample from 15 control (*Helicobacter*-free) mice.

In a previous study, we reported the construction and screening of an *H. pylori* expression library in order to identify potential candidates for subunit

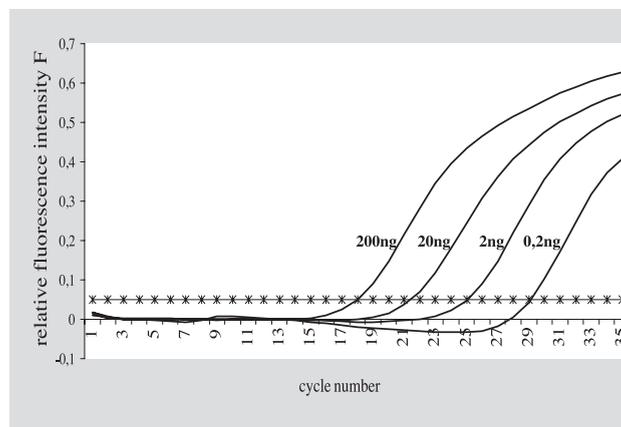
vaccine antigens (Łazowska et al., 2000). There were 114 positive clones isolated. One of them, selected in screening with both human sera and rabbit antiserum, was *urease beta* subunit and its nucleotide sequence was used for construction of the bacterial expression vector. There were no detectable antigenic differences between *rUreB* produced by *E. coli* containing pGEX 4T-1-*ureB* plasmid and the protein expressed by *E. coli* carrying *ureB* recombinant lambda ZAP Express clones.

Next, the level of infection was assessed in mice prophylactically immunized with *rUreB* or *H. pylori* whole-cell sonicate antigens. Seven out of ten mice that underwent prophylactic immunization with *rUreB* plus cholera toxin appeared to be protected against experimental *H. pylori* infection, as revealed by the real-time PCR. In three other mice, real-time PCR confirmed low level of *H. pylori* infection, showing bacterial cell densities ranging between 326 and 919 cells/100 ng of mouse DNA (Fig. 4). In all eight mice that were prophylactically immunized with *H. pylori* sonicate plus cholera toxin, real-time PCR showed bacterial DNA corresponding to bacterial cells in the range between 97 and 710 cells (Fig. 4). The median bacterial load in the stomachs of mice immunized with *H. pylori* sonicate was reduced to 5% of that of experimentally infected mice without any prophylactic immunization.

In noninfected BALB/c mice, the presence of *Helicobacter*-like bacteria that are urease-positive can cause false-positive results in the rapid urease (CLO) test (Ruiz-Bustos et al., 2000). In contrast, the CLO tests were negative after incubation of up to 24 h in all 15 *Helicobacter*-free C57BL/6. Thus, the results from real-time PCR could be compared with urease activity assays.

In each sham-vaccinated experimentally infected mouse, a rapid urease test was positive (in most cases

A.



B.

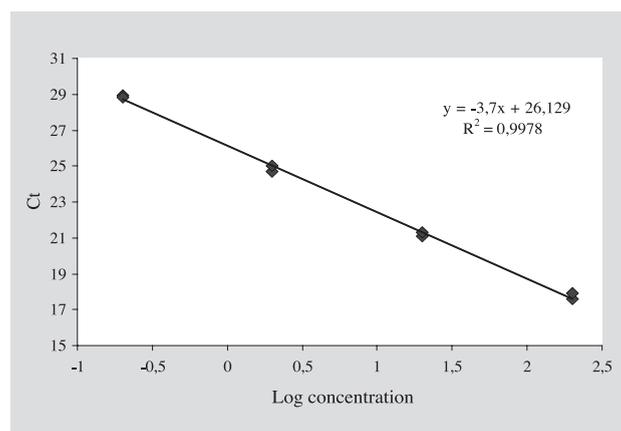


Fig. 3. Amplification plots (A) and standard curve (B) for the quantification of the *GAPDH* host pseudogenes showing C_T values plotted versus the log of the initial 10-fold dilution series of mouse genomic DNA (0.2 to 200 ng).

it was rapidly positive). However, the low infection level was confirmed by CLO test only in one out of eight mice immunized with *H. pylori* whole-cell sonicate and in two out of three mice immunized with purified *rUreB*. The rapid urease test was positive only in those immunized mice that exhibited the highest amount of *H. pylori* DNA, as detected by real-time PCR.

The gold standard method for the detection of *H. pylori* infection is bacterial culture (Leodolter et al., 2001). Although it is a very specific method in experienced hands, its sensitivity is limited because

of technical difficulties, especially at low bacterial quantities. Histological identification of *H. pylori* is also highly sensitive and specific (Leodolter and Megraud, 2001; Hirschl and Glupczynski, 1999; van Zwet and Megraud, 1998;Leodolter et al., 2001) and allows for semiquantitative infection determination. However, this method requires experienced pathologists, and inappropriate stains can decrease the histological assessment. The rapid urease test can reflect a level of *H. pylori* infection in the stomach comparable to that assessed by histological examination (Furuta et al., 1996).

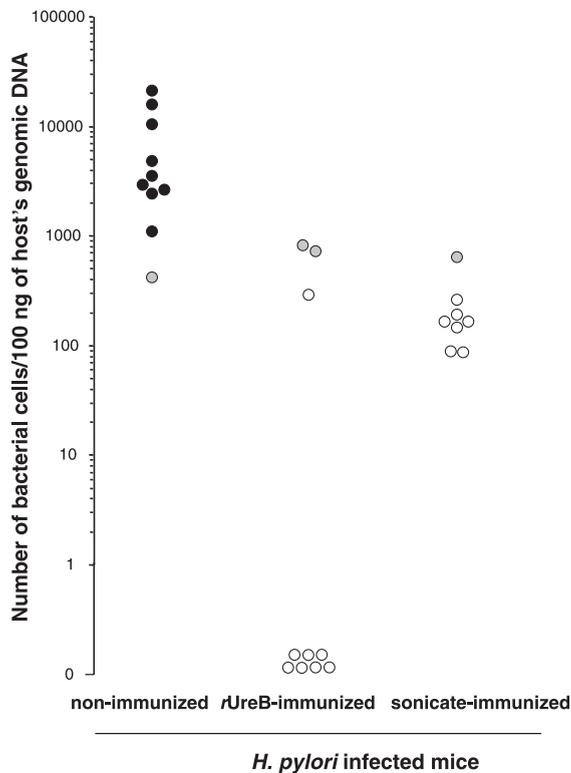


Fig. 4. Gastric colonisation level of *H. pylori* in sham-immunized and prophylactically immunized mice; comparison of real-time PCR-based detection assay targeting the 26-kDa *Helicobacter* species-specific antigen (*SSA*) gene with the rapid urease test (CLO test). Shown are numbers of bacterial cells per 100 ng of host's genomic DNA. Open circles—negative CLO test. Closed circles—positive CLO test. Semi-closed circles—positive CLO test but markedly slower than CLO test in sham-vaccinated experimentally infected mice.

Oral immunization of C57BL/6 mice with *H. pylori* antigens resulted in a significant reduction of the levels of *H. pylori* colonisation after experimental infection. Low-level infection could be determined by the real-time PCR but not by the rapid urease test, and this comparison indicates that in prophylactically immunized mice low colonisation levels of experimental *H. pylori* infection can be below the limit of detection for urease test. Not unexpectedly, our studies confirmed the previously published observation (Kleanthous et al., 1998) that the gastric urease assay is relatively insensitive in detecting *H. pylori* in gastric mucosa. Therefore, this test may indicate a complete protection even in a case of residual infection. In

contrast, sterilizing immunity can be easily discriminated from incomplete protection by using quantitative real-time PCR assay.

In our SYBR Green I real-time PCR assay the detection limit was 10 fg (corresponding to 5 bacterial cells) per 200 ng of host's DNA. Interestingly, the same detection limit was found for *H. hepaticus* when TaqMan real-time PCR was used (Ge et al., 2001). This sensitivity is much higher than the sensitivity of other diagnostic tests available for the diagnosis of *H. pylori* and should be sufficient for use in both experimental and clinical studies. However, although the sensitivity of the PCR method is very high, its specificity may not be enough in clinical studies because of endoscopic contamination with *H. pylori* DNA. Notably, the higher sensitivity of PCR increases the probability of a false-positive result due to sample contamination and, therefore, real-time PCR may have limitations in clinical practice. On the other hand, as shown in this study, real-time PCR can easily detect insufficient protection of the applied vaccination. Thus, the developed method for a quantitative analysis of *H. pylori* in host's stomach may be very useful in experimental studies evaluating new anti-*H. pylori* drugs and vaccines.

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