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Note

Detection of the *Helicobacter pylori dupA* gene is strongly affected by the PCR design

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ABSTRACT

The *Helicobacter pylori* virulence gene *dupA* is usually detected by PCR, but the primer binding sites used are highly variable. Our newly designed qPCR against a conserved region of *dupA* was positive in 64.2% of 394 clinical isolates while the positivity rate of the commonly used PCRs ranged from 29.9% to 37.8%.

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Infection with the human pathogen *Helicobacter pylori* can result in gastroduodenal disorders, ranging from chronic gastritis to peptic ulcer and gastric cancer (Kusters et al., 2006). Lu et al. described the duodenal ulcer promoter gene A (*dupA*) to be associated with *H. pylori* induced ulcer formation (Lu et al., 2005). Several reports confirmed these associations, but others do not find any associations (Abadi et al., 2012; Arachchi et al., 2007; Gomes et al., 2008; Hussein, 2010; Queiroz et al., 2011). Takahashi et al. (2013) pointed out considerable sequence variation between *dupA* genes and claimed that only a specific allele (the intact long-type dupA) is associated with ulcer induction, but their evidence still (partially) relied on *dupA* PCR. Given the high degree of sequence variation within *dupA* one might argue that this might result in primer mismatches leading to erroneous PCR results. The aim of this study was to determine the effect of an improved PCR design on the detection of *H. pylori dupA*.

From a collection of all *H. pylori* strains cultured from patients that had consulted the Zaans Medical Center, Zaandam, The Netherlands, between 2005 and 2007 we selected the first 400 strains. DNA was isolated by standard automated DNA extraction using the MagNA Pure 96 DNA (Roche Diagnostics, Almere, the Netherlands). All PCR reactions always included the fully sequenced reference strains J99 and 26695 (ATCC 700824 and 700392 respectively) as positive and negative controls, respectively. To confirm the *H. pylori* nature and to check for the quality of the isolated DNA a PCR reaction with the *H. pylori* specific EHC-U/L PCR was performed (Li et al., 1995). From 6/400 strains we could not obtain a ECHC-U/L PCR product, indicating insufficient DNA quality and/or PCR inhibition, leaving 394 *H. pylori* strains.

A PCR targeting a conserved area of *dupA* were designed based on an alignment of all 221 *dupA* gene sequences present in the NCBI DNA database as of January 2013 (Table 1). A Primer-Blast analysis (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) predicted that these primers would detect 220/221 of the NCBI database sequences that contain the corresponding fragment. During the revision we were asked to perform a similar analysis for the other PCR reactions but as the annealing temperature of these reactions is not standard we had to perform a manual analysis. This predicted an in silico positivity rate for the jhp0917, jhp0918, dupA, and AR-dupA PCR of 65.9%, 33.5%, 26.3%, and 98.6%, respectively (Table 1). Considering that 254/379 (67.0%) of the full *H. pylori* genomes in the NCBI database carry a *dupA* gene the ratios of *dupA* positive PCRs observed among the 396 strains are in line with these predictions (Table 1). The *jhp0918* PCR showed the lowest *dupA* detection rate (29.9%) while the newly designed *AR-dupA* PCR had the highest positivity rate. It is unlikely that the increased *dupA* detection rate is a consequence of this being a real time PCR because when the qPCR products were analyzed on a gel they had similar band intensities as the other PCRs. All four PCR assays detected a different subset of strains with 30/394 (7.6%) of the strains being only positive in the *AR-dupA* PCR. Sequence analysis of these 30 exclusively *AR-dupA* PCR products confirmed that they actually represented true *dupA* positives. The *jhp0917* PCR was positive in 149/394 (37.8%) of the samples and the *jhp0918* PCR in 118/394 (29.9%). Using the

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Table 1
Details of PCR assays used in the current study.

PCR name	Oligonucleotide characteristics[a]			PCR annealing temperature[b]	Size of PCR fragment	Fraction of *dupA* in NCBI predicted to be PCR positive[d]	Actual *dupA* PCR positives in 394 Dutch isolates	Original PCR design
	Orientation	Oligonucleotide composition	Tm[c]					
jhp0917	Fw	5′-TGGTTTCTACTGACAGAGCGC-3′	60.3 °C	59 °C	307 bp	118/179 (65.9%)	149 (37.8%)	Lu et al. (2005)
	Rev	5′-AACACGCTGACAGGACAATCTCCC-3′	64.9 °C					
jhp0918	Fw	5′-CCTATATCGCTAACGCGCGCTC-3′	63.8 °C	58 °C	276 bp	69/206 (33.5%)	118 (29.9%)	Lu et al. (2005)
	Rev	5′-AAGCTGAAGCGTTTGTAACG-3′	57.3 °C					
dupA	Fw	5′-TAAGCGTGATCAATATGGAT-3′	51.8 °C	56 °C	350 bp	59/224 (26.3%)	132 (33.5%)	Nguyen et al. (2010)
	Rev	5′-TGGAACGCCGCATTCTATTA-3′	57.4 °C					
AR-dupA	Fw	5′-CATGGCGTTTCAAAAAATATCTCAA-3′	57.4 °C	60 °C	112 bp	284/288 (98.6%)	253 (64.2%)	Current study
	Rev	5′-TTCATCAGTATCTTTTGTGGGGTA-3′	57.6 °C					
	Probe	FAM-GGCAACCTTTCTCAAGTGATTATC-BBQ	58.1 °C					

[a] All oligonucleotides were produced by TIB MOLBIOL GmbH, Berlin, Germany. FAM: 6-carboxyfluorescein and BBQ: BlackBerry Quencher. Fw: forward primer, Rev: reverse primer and Probe: hydrolysis probe.
[b] PCR conditions (denaturation, annealing, and extension times and temperatures) for the three commonly used dupA PCR reactions (jhp0917, jhp0918 and dupA) and the EHC-U/L positive PCR control were performed as standard endpoint PCR reactions as described in the original references(Lu et al., 2005; Nguyen et al., 2010 and Li et al., 1995 respectively). PCR conditions for the newly designed real-time PCR (AR-dupA) were: 95 °C for 10 min (pre-incubation), 42 amplification cycles consisting of 95 °C for 20 s and 60 °C for 55 s. All PCR amplification reactions were performed in a Roche Light Cycler 480 II (Roche Diagnostics, Almere, the Netherlands).
[c] Tm of primers was calculated with Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the default settings of the program.
[d] All sequences spanning the jhp0917, jhp0918, dupA, and AR-dupA PCR products were retrieved from NCBI database on June 30th 2014. Fraction represents the total number of predicted positives/total number of sequences in the database that contained both full forward and reverse primer binding sites. Criteria for predicting a PCR to be negative were that either the forward or reverse primer (or both) contained: 1. Primer-mismatches resulting in a Tm > 5 °C below the annealing temperature of the PCR reaction, or 2. one or more mismatches in the five 3′ end nucleotides of a primer, or 3. three or more mismatches in a primer.

recommendation of the original designers of the *jhp0917* and *jhp0918* PCRs to report the *dupA* status only when both the *jhp0917* and *jhp0918* PCRs were concordant only for 254/394 isolates (64.4%) a *dupA* status could be obtained resulting in a combined *dupA* positivity rate of 41/254 (16.1%).

The presence of *dupA* is considered a true virulence factor with no linkage to other classical virulence factors like *cagA*, *vacA*, and *iceA* (Yamaoka, 2012). However not all agree that the presence of *dupA* is associated with duodenal ulcers (Arachchi et al., 2007; Argent et al., 2007; Imagawa et al., 2010). As far as we know no-one has actually tested the technical limitations of the PCR assays used to detect the *dupA* status as a potential reason for these discrepant results. *H. pylori* is a genetically variable bacterium and especially at the plasticity region where the *dupA* gene is located is known to display substantial genomic diversity (Sugimoto et al., 2012). An in silico evaluation of the *dupA* gene sequences from the public DNA databases revealed significant mismatches of the different *dupA* primers that have been used in the published studies (Table 1). Indeed the prevalence of *dupA* among the *H. pylori* strains was significantly higher when using a newly designed primer set that targeted conserved areas of the *dupA* gene, indicating that the *dupA* PCR assays that have been used in the published studies probably missed some *dupA* positive isolates. According to our in silico prediction our PCR will detect most (98.6%) of the *dupA* genes and this seems to be confirmed by the findings in our population; it is not unthinkable that it may miss specific *dupA* variants present in other geographical areas. In conclusion, our findings suggest that previous results on the increased prevalence of *dupA* in patients suffering from *H. pylori* induced ulceration should be interpreted with caution. More likely these papers reported the association of a specific *dupA* allele instead of the actual presence of the *dupA* gene. Thus rather than disputing or confirming the association between the presence of *dupA* and different gastroduodenal diseases these reports tested a relationship between a specific *dupA* allele and disease. Since various primer sets were used these different PCR assays probably tested for different *dupA* alleles and can thus not be compared to each other. Unfortunately, we do not have clinical data related to the strains from our collection, thus we cannot check the association between the specific disease outcome and a positive *dupA* PCR. For other *H. pylori* virulence factors such as *vacA* it is not the presence of the *vacA* gene but rather the presence of specific alleles (s_1/s_2, m_1/m_2) that is associated with a different risk on inducing *H. pylori* associated disease (Atherton et al., 1999). The challenge would be to identify these disease specific *dupA* alleles and to design allele specific primers for their detection.

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