Clinical Comparison of Multiplex TEM-qPCR to Histopathology for Detection of *Helicobacter pylori* in the Gastric Mucosa

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Abstract

Helicobacter pylori is not commonly cultured for diagnosis and current diagnostic methods do not offer antibiotic susceptibility. A PCR method was therefore developed to identify *H. pylori* in gastric biopsies and to determine genetic resistance to clarithromycin. This study evaluated the clinical accuracy of a target enriched multiplex-qPCR *Helicobacter pylori* panel (TEM-qPCR) compared to histopathology. All biopsies were also tested for presence of Epstein-Barr virus (EBV) by qPCR to assess correlation with *H. pylori*.

Gastric biopsies were collected from symptomatic patients (n=93, mean age 54, 55% female, 48% on acid lowering medications) and submitted for histopathology and TEM-qPCR. The Diatherix TEM-qPCR HP panel tests for the urease gene and point mutations in *H. pylori* conferring clarithromycin resistance. The limit of detection for TEM-qPCR is less than 10 cfu/mL with no cross-reactivity observed among 90 organisms.

H. pylori was detected by histopathology in 10/93 samples and by TEM-qPCR in 12/93 with 100% sensitivity and 97.6% specificity before discrepancy resolution. Discrepancies were resolved as true positives using an orthogonal *H. pylori* detection method. Of all samples, one was predicted to be a mixed clarithromycin susceptible and resistant *H. pylori* population, and one sample was predicted to be clarithromycin resistant. EBV was detected in 24% of tested samples with 75% prevalence of EBV co-detected with *H. pylori* (*p=<0.0001*).

In conclusion, the novel TEM-qPCR HP panel is more sensitive than histopathology for detecting *H. pylori* in gastric biopsies and provides detection of the genetic determinants of clarithromycin resistance. EBV detection in gastric mucosa was correlated to *H. pylori* presence.

Note: Abstract updated to reflect the inclusion of additional patients.

Introduction

- *H. pylori* treatment frequently consists of a triple therapy including clarithromycin, resistance to which greatly reduces eradication rates[1]
- Clarithromycin resistance rates are rising in North America and Europe[1]
- *H. pylori* is fastidious and difficult to culture complicating matters to obtain sensitivity, relying instead upon regional prevalence of treatment failure or molecular methods[1]
- A multiplex qPCR panel was developed to detect *H. pylori* and the most common point mutations conferring clarithromycin resistance
- A clinical comparison study between the qPCR panel and histological staining of gastric biopsies for *H. pylori* was conducted to assess diagnostic sensitivity
- Biopsies were additionally tested by PCR for prevalence of Epstein-Barr virus co-detected with *H. pylori*

Materials & Methods

Study Protocol
- Study protocol was approved by Sterling Institutional Review Board (Atlanta, GA)
- Study inclusion criteria included age >18 years, symptoms of active peptic ulcer disease or dyspepsia, clinical suspicion of *H. pylori* infection with esophagogastroduodenoscopy (EGD) and gastric biopsy ordered, abstinence from antibiotics for 14 days previous to EGD, and willing and able to provide informed consent
- Study was coordinated by AIG Research Services (Hermitage, TN)

Sample Collection
- Two gastric biopsies were collected during esophagogastro-duodenoscopy (EGD) from the greater angularis and/or greater curvature of the antrum by Associates in Gastroenterology, LLC (Hermitage, TN)
- Biopsies were placed in sterile 1X phosphate buffered saline (PBS) and stored at -80ºC until DNA extraction

DNA Extraction
- DNA was extracted from biopsies with DNA Extract All (ThermoFisher Scientific, Foster City, CA)
- Briefly, 100µL of lysis solution was added to biopsy samples and incubated at 95ºC for 3 minutes. Samples were cooled to room temperature and 100µL of DNA stabilization solution was added before being stored at 4ºC for short-term storage and -80ºC for long term storage

Target Enriched Multiplex Quantitative PCR (TEM-qPCR)
- A five probe TaqMan™ (ThermoFisher, Waltham, MA) multiplex nested qPCR panel was developed
- A novel cycling protocol was developed involving a target enrichment pre-amplication PCR, integration of tagged primer sequences, dilution of pre-amp, followed by 40 cycles of qPCR with probes and super primers corresponding to the tagged primer sequences

Table 1. *H. pylori* qPCR Panel Targets

Target	Fluor/ Quencher	Emission (nm)
H. pylori ureA (urease subunit)	JUN/ QSY	617
H. pylori 23S WT* (wild type)	VIC/ QSY	551
H. pylori 23S A2142G*	NED/ MGB	575
H. pylori 23S A2143G*	FAM/ MGB	517
Internal Control	CY5/ Iowa Black	668

*Confers susceptibility or resistance to clarithromycin

Epstein-Barr Virus Nested qPCR
- A nested singleplex qPCR assay was developed to detect Epstein-Barr Virus (EBV) with a similar amplification technique to TEM-qPCR though not using super primers and relying instead on differing melt temperatures of nested primers to accomplish an integrated pre-amp

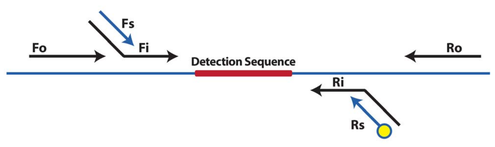
Materials & Methods *(continued)*

Target Enriched Multiplex End-Point PCR (TEM-PCR) with Barcoded Magnetic Bead detection
- An orthogonal approach for discrepancy analysis was developed targeting the urease accessory protein gene, *ureE,* with a different amplification approach, and a different detection method
- Target Enriched Multiplex End-Point PCR is a nested PCR approach with low concentration gene-specific primers, tagged inner primers, and universal super primers
- Cycling protocol involved a target enrichment step, a tagging step, and an exponential amplification step with super primers
- Barcoded magnetic beads (Applied Biocode, Santa Fe Springs, CA) were covalently coupled to a detection sequence corresponding to the *ureE* amplicon and detected with the Biocode 2000 (Applied Biocode) instrument

Table 2. Oligonucleotide sequences used for orthogonal discrepancy resolution

Target Gene		Oligonucleotide	Sequence (5'-3')
ureE	Urease Accessory Protein	ureE Fo1	GCTTGGATGTGAATGACTTCAG
		ureE Fi1	TCAGAATCCAAGATATTAACGG
		ureE De2	CTTGGGAGCGTCTTTAAGG
		ureE Ri1	AGGCAAAGACATAGCCATACG
		ureE Ro1	TGTGGATTTGGAATGGTTTG

Figure 1. TEM-PCR™ Scheme. Low concentration nested gene-specific primers are (Fo – forward out; Fi – forward in; Ri – reverse in; and Ro – reverse out) designed to enrich the targets during the initial PCR cycles. Later in the procedure, a pair of universal SuperPrimers (Fs and Rs) is used to amplify all targets. The Rs primer is labeled with biotin for subsequent detection.



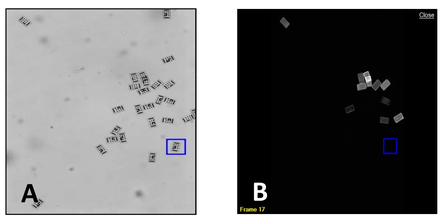


Figure 2. Barcoded magnetic bead detection with Applied BioCode reader. (A) Bright-field image. (B) Fluorescence image. Hybridization of amplicons is determined by binding of fluorescent dye (SA-PE) to biotin label of amplicons hybridized to the beads.

Results

Table 3. Comparison of detection results for *H. pylori* between TEM-qPCR and histological staining before resolution of discrepancies

Assay	Histology Staining		Assay performance (95% confidence interval)			
	Detected	Not Detected	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
TEM-qPCR						
Detected	10	2				
Not Detected	0	81	**100**** (69-100)	**97.6**** (92-100)	**83.3***** (56-95)	**100***** (94-100)
Total	**10**	**83**				

**Confidence intervals for sensitivity and specificity are "exact" Clopper-Pearson confidence intervals.[2]
***Confidence intervals for the predictive values are the standard logit confidence intervals given by Mercaldo et al. 2007[3]

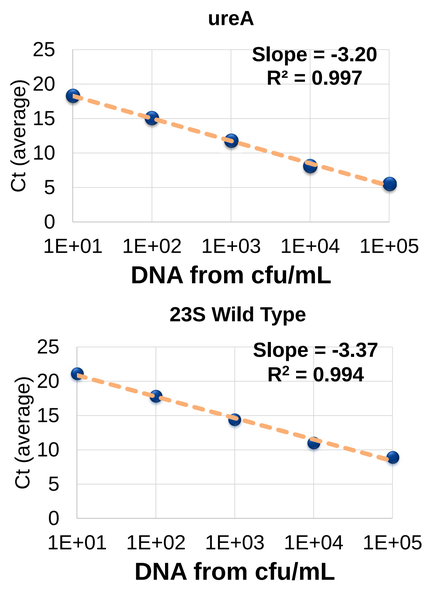


Figure 3. Standard curves of urease and clarith-romycin susceptibility targets. *ureA* and 23S wild type targets demonstrated sensitivity down to 10 cfu/mL. *ureA* has a linear quantitation range from 10 to 100,000 cfu/mL whereas the 23S WT target has a linear quantitation range from 10 to 10,000 cfu/mL. qPCR was setup with four replicates per conc-entration. Average standard deviation is 0.14 for *ureA* and 0.17 for 23S WT.

Table 4. Resolution of discrepancies comparing TEM-qPCR results to *ureE* end-point assay

Assay	Diatherix Eurofins TEM-qPCR *H. pylori*		% Agreement
	Detected	Not Detected	
ureE TEM-PCR			
Detected	12	0	**100**
Not Detected	0	81	
Total	**12**	**81**	

Results *(continued)*

Table 5. Correlation between Epstein-Barr Virus Co-detection with *H. pylori*

Assay	Diatherix Eurofins TEM-qPCR *H. pylori*		*p= <0.0001***
	Detected	Not Detected	
EBV qPCR			**Total**
Detected	9	14	**23**
Not Detected	3	67	**70**
Total	**12**	**81**	**93**

****P value calculated with Fisher's exact probability test

Table 6. Prevalence of genetic determinants of clarithromycin resistance

Genotype	n	Percent
Wild type	10	83
A2142G	0	0
A2143G	1	8
Mixed	1	8

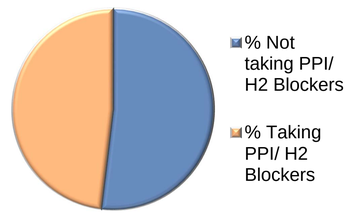


Figure 4. 48% of participants were currently on proton pump inhibitors (PPI) or H2 blockers

Table 7. Summary of Epstein-Barr Virus (EBV) positive gastric biopsies

EBV Result	Age	*H. pylori* Histology Result	Histology Stain(s)	Pathology Lab Site	*H. pylori* TEM-qPCR Result	*ureE* Result
DET	78	DET	Diff-Quick, Alcian Blue	1	DET	DET
DET	82	NOT DET	Diff-Quick, Alcian Blue	1	NOT DET	NOT DET
DET	33	DET	Diff-Quick, Alcian Blue	1	DET	DET
DET	67	NOT DET	Diff-Quick, Alcian Blue	1	NOT DET	NOT DET
DET	53	DET	Silver	2	DET	DET
DET	28	NOT DET	Silver	2	NOT DET	NOT DET
DET	48	NOT DET	Diff-Quick, Alcian Blue	1	NOT DET	NOT DET
DET	71	NOT DET	Diff-Quick, Alcian Blue	3	NOT DET	NOT DET
DET	28	NOT DET	Diff-Quick, Alcian Blue	3	NOT DET	NOT DET
DET	83	NOT DET	Diff-Quick, Alcian Blue	3	NOT DET	NOT DET
DET	38	DET	Silver	2	DET	DET
DET	80	NOT DET	Diff-Quick, Alcian Blue	1	NOT DET	NOT DET
DET	68	NOT DET	Diff-Quick, Alcian Blue	1	NOT DET	NOT DET
DET	48	DET	Immunoperoxidase, Alcian Blue	4	DET	DET
DET	58	DET	Silver	2	DET	DET
DET	70	NOT DET	Diff-Quick, Alcian Blue	3	NOT DET	NOT DET
DET	72	DET	Diff-Quick, Alcian Blue	3	DET	DET
DET	42	DET	Diff-Quick, Alcian Blue	1	DET	DET
DET	53	NOT DET	Diff-Quick, Alcian Blue	3	NOT DET	NOT DET
DET	41	NOT DET	Silver	2	NOT DET	NOT DET
DET	58	DET	Diff-Quick, Alcian Blue	3	DET	DET
DET	68	NOT DET	Diff-Quick, Alcian Blue	1	NOT DET	NOT DET
DET	23	NOT DET	Immunoperoxidase, Alcian Blue	4	NOT DET	NOT DET

DET- *H. pylori* detected NOT DET- *H. pylori* not detected

1- Associates in Gastroenterology 2- GI Pathology
3- Pathologists' Laboratory PC 4- Gastro One

Conclusions

- A multiplex qPCR method was developed for the detection of *H. pylori* from gastric biopsies and the detection of the most common genetic determinants of clarithromycin resistance with an analytical sensitivity less than 10 cfu/mL
- The Diatherix Eurofins *H. pylori* panel was found to be highly correlative with results from histological staining including in the presence of PPI's
- The orthogonal *ureE* confirmation method was determined to have 100% agreement with initial results from TEM-qPCR
- Two discrepancies were resolved as false negatives for histology demonstrating the increased sensitivity of TEM-qPCR
- The analytical specificity of the Diatherix Eurofins *H. pylori* panel was tested with 90 microorganisms associated with the oral and gastrointestinal tract and was not found to cross-react with any of them. The diagnostic specificity was demonstrated to be very high (97.6%) before discrepancy resolution and 100% after
- Correlation between Epstein-Barr Virus co-detection with *H. pylori* was found to be extremely significant (*p= <0.0001)* by Fisher's exact test. It is assumed EBV status is due to chronic latency. Age or severity of histological observation were not correlative to EBV detection
- EBV has previously been found to increase *H. pylori* virulence[4], and was found to be present in 75% of samples alongside *H. pylori* compared to 17% in the absence of *H. pylori* in this study
- 2/12 Samples with *H. pylori* detected are predicted to have clarithromycin resistance by the panel. The number of samples is too low to determine statistical significance

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