Stool Microbiome Reveals Diverse Bacterial Ureases as Confounders of Oral Urea Breath Testing for *Helicobacter pylori* and *Mycobacterium tuberculosis* in Bamako, Mali

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Abstract

Detection of bacterial urease activity has been utilized successfully to diagnose *Helicobacter pylori (H. pylori)*. While *Mycobacterium tuberculosis (M. tb)* also possesses an active urease, it is unknown whether detection of mycobacterial urease activity by oral urease breath test (UBT) can be exploited as a rapid point of care biomarker for tuberculosis (TB) in humans. We enrolled 34 individuals newly diagnosed with pulmonary TB and 46 healthy subjects in Bamako, Mali and performed oral UBT, mycobacterial sputum culture and *H. pylori* testing. Oral UBT had a sensitivity and specificity (95% CI) of 70% (46–88%) and 11% (3–26%), respectively, to diagnose culture-confirmed *M. tb* disease among patients without *H. pylori*, and 100% sensitivity (69–100%) and 11% specificity (3–26%) to diagnose *H. pylori* among patients without pulmonary TB. Stool microbiome analysis of controls without TB or *H. pylori* but with positive oral UBT detected high levels of non-*H. pylori* urease producing organisms, which likely explains the low specificity of oral UBT in this setting and in other reports of oral UBT studies in Africa.

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Keywords

oral urease breath test; urease; TB; *H. pylori*

INTRODUCTION

Despite the availability of inexpensive and effective treatment, tuberculosis (TB) still accounts for millions of deaths worldwide [1, 2]. The highest burden of disease is borne by developing countries where disease prevalence correlates with malnutrition, densely-populated communities, lack of access to affordable health care, and synergy with HIV co-infection. Sputum smear microscopy, which is more than 100 years old and lacks sensitivity, remains the most widely used diagnostic tool. Other tests with improved sensitivity, such as sputum culture and molecular based testing are often either technically difficult or too expensive for low-income countries with endemic TB. There is therefore a pressing need for the development of inexpensive and sensitive tools for the diagnosis of active TB in a point of care setting. Indeed, a recent report by UNITAID and the WHO has emphasized the need for a rapid, point-of-care screening tool for TB in resource-poor settings [3,4].

Ureases are found in numerous microbial species, including bacteria, but are not present in humans [5,6]. Assays that detect bacterial urease activity have an essential role in the diagnosis and clinical management of *Helicobacter pylori* (*H. pylori*) gastritis [7]. In *H. pylori* infection, the bacterial urease catalyzes the hydrolysis of urea into ammonia and carbon dioxide (CO_2), which increases stomach pH and allows local survival of *H. pylori*. Carbon dioxide, a byproduct of this chemical reaction, is exhaled, where it can be detected in breath samples [7,8]. This phenomenon has been exploited for use in diagnostic tests for *H. pylori,* including the oral urease breath test (UBT) and rapid urease test (RUT) [9,10].

The oral UBT administers a dose of labeled ^{13}C-urea by mouth; if urease is present, it hydrolyzes the stable isotope labeled urea, and $^{13}CO_2$ is produced [11]. The $^{13}CO_2$ to $^{12}CO_2$ ratio in exhaled air is readily measured with portable infrared spectrometers, and the entire procedure can be completed within 30 minutes [12]. The test was approved by the Food and Drug Administration (FDA) for the diagnosis of *H. pylori* infection in 1997 [13], and it is also used to confirm eradication after antibiotic therapy [14]

Like *H. pylori*, *Mycobacterium tuberculosis* (*M. tb*) also possesses an active urease [15]. *In vitro* urease testing has long been used for mycobacterial species identification [16], but whether *in situ* detection of mycobacterial urease activity can be exploited for clinical diagnosis or monitoring of *M. tb* treatment in humans has not been explored. As part of our efforts to develop a breath test for TB, in preclinical studies, we found that rabbits with pulmonary TB rapidly convert [^{13}C]-labeled urea into $^{13}CO_2$, and that signal can be detected by UBT in a matter of minutes [17–19]. Furthermore, the UBT signal diminished as the rabbits were exposed to antimicrobial therapy and then increased again after therapy was halted, which suggested that the UBT may have a promising role in monitoring response to therapy [18]. Extrapolating from those data, lack of UBT signal inhibition during antibiotic treatment may be a rapid indicator of treatment failure among individuals with drug-resistant TB who are being treated with inadequate drug regimens. A study on TB and *H. pylori* co-

infection in Iran showed the disappearance of UBT signal when patients were treated with standard TB therapy (without concomitant *H. pylori* treatment), which suggests that *M. tb* infection may contribute to the positivity of UBT [20].

Both *H. pylori* and *M. tb* are widely endemic infections in Mali with a prevalence of 92 per 100,000 for TB and an estimated percentage of *H. pylori* infection ranging from 41 to 97% [2, 21]. As co-infection with *H. pylori* and *M. tb* may confound results of the oral UBT, we evaluated the sensitivity and specificity of oral UBT to diagnose active TB and *H. pylori* in patients who were co-infected with *H. pylori* and *M. tb*, mono-infected with *M. tb*, and mono-infected with *H. pylori*. Patients who were double negative for both TB and *H. pylori* infections were used as negative controls.

METHODS

Study Population

From June of 2011 to December 2012, individuals who were 18 years of age or older were consecutively recruited from six referring health centers of Bamako and Point-G Hospital of Bamako, Mali. A total of 34 individuals who were newly diagnosed with active pulmonary TB (with or without *H. pylori* infection) and 46 healthy volunteers (with or without *H. pylori* infection) were enrolled and completed the study protocol. Participation was voluntary, written informed consent was obtained prior to enrollment, and participation did not alter provision of standard medical care to patients. This study was approved by the Institutional Review Board of the US National Institutes of Allergic and Infectious Diseases (NIAID) and the Ethics Committee of the Faculty of Medicine, Pharmacy and Dentistry (FMPOS) of the University of Sciences, Techniques and Technology of Bamako (USTTB). It was registered with ClinicalTrials.gov, identifier NCT01301144.

Study Procedures

All participants underwent a physical exam and a baseline evaluation of his or her medical history. Newly diagnosed patients with pulmonary TB were enrolled on the basis of two positive acid-fast bacilli (AFB) sputa assessments and prior to initiation of any TB drugs. Sputum microscopy assessments were performed by both Ziehl-Neelsen staining at local referral centers and by auramine rhodamine staining using fluorescence microscopy performed at the central SEREFO BSL-3 laboratory. Active pulmonary TB was later confirmed by mycobacterial culture in both MGIT and on solid agar 7H11 plates. To meet enrollment criteria, the apparently healthy volunteers had to be without any signs or symptoms of active TB. This included lack of documented fever, cough or weight loss, and a microbiological assessment that included two sputum specimens that were negative for AFB. The healthy controls were all ultimately confirmed to be TB culture negative. *H. pylori* stool antigen testing was performed using ImmunoCard HPSA® (Meridian Diagnostics). Additional study testing included HIV-1/2 serology (Determine® HIV-1/2, Abbott) and pregnancy testing for female participants. Key exclusion criteria included: use of any antibiotic (including TB drugs), proton pump inhibitors or bismuth containing preparations in the 15 days prior to study enrollment, HIV positivity and pregnancy.

TB and *H. pylori* Case Definitions

Pulmonary TB cases were defined by positive mycobacterial culture by MGIT and/or 7H11 culture. *H. pylori* stool antigen testing was used to define the patient's *H. pylori* status. Patients were enrolled on the basis of initial TB and *H. pylori* testing and were later assigned to one of the four following diagnostic categories on the basis of their study mycobacterial culture and *H. pylori* stool antigen results: (*1*) active pulmonary TB with *H. pylori* co-infection (TB+/HP+, Group 1); (*2*) active pulmonary TB without *H. pylori* co-infection (TB+/HP−, Group 2); (3) no apparent TB but *H. pylori* infection (TB−/HP+, Group 3); and (4) no apparent TB and no *H. pylori* infection (TB1/HP−, Group 4)(Figure 1). This study included a total of 80 study subjects distributed in the four cited groups (Table 1).

Urea Breath Testing

BreathTek™ UBT Collection Kit (Otsuka America Pharmaceutical Co., Ltd.) was utilized for oral UBT in this study. Subjects ingested 75 mg of a synthetic ^{13}C-urea (Pranactin®-Citric) reconstituted with water. Measurement of the ratio of $^{13}CO_2$ to $^{12}CO_2$ in a post-dose breath sample was performed at baseline (before the Pranactin®-Citric) and at three different time points (10, 20, and 45 minutes) following oral urea administration. The measured difference between the ratios of $^{13}CO_2$ to $^{12}CO_2$ values before and after administration of Pranactin-Citric solution is reported as Delta over Baseline (DOB), with a value greater than or equal to 2.4-fold above baseline considered a positive test. The study time points were selected as optimal based on a pilot study. For patients with presumed pulmonary TB (AFB positive at the time of enrollment), UBT sampling was performed on two consecutive days prior to initiation of any TB antimicrobials and mean DOB values were used. Healthy controls were assessed only once.

Stool Microbiome Assessment

Stool samples (approximately 5g) were collected from 10 study participants who had a positive UBT, but no clinical or laboratory evidence of either pulmonary TB or *H. pylori*. These participants tested negative for *H. pylori* stool antigens (ImmunoCard HPSA®, Meridian Diagnostics) as well as negative for *H. pylori* serum antibodies (ImmunoCard *H. pylori* IgG®, Meridian Diagnostics). Whole metagenome shotgun sequencing was performed at the University of Maryland Institute for Genome Sciences using the Illumina HiSeq2000 platform to generate 101bp paired-end reads for analysis. Microbiome analysis was performed at The Broad Institute, Boston, MA.

Data analysis

The sensitivity and specificity along with 95% confidence intervals of oral UBT for diagnosis of TB and *H. pylori* infection was calculated using Stata version 12.1, with a UBT threshold for positivity being a DOB value of 2.4-fold above baseline. The organismal composition of microbial communities from stool was determined using alignment-based approaches that employed either a database of unique microbial genomes [22], or marker genes representing organisms at various taxonomic levels [23]. Functional metabolic pathways were predicted using the HUMAnN pipeline [24], and the relative abundance of

predicted ureases was compared to the abundance found within stool samples from the Human Microbiome Project focused on healthy individuals from the United States [25].

RESULTS

Patient Population

Of the 80 volunteers enrolled in this study, 34 (42.5%) were confirmed to have pulmonary TB on the basis of a positive sputum culture (TB+), whereas 46 (57.5%) were mycobacterial culture negative (TB−) and were used as healthy comparators. Ultimately, 24 (30%) patients were determined to have a positive stool antigen test for *H. pylori,* and were considered actively infected with *H. pylori* (HP+), whereas, 56 (70%) had negative stool antigen testing and presumed *H. pylori* uninfected (HP−). When patients were distributed into groups on the basis of both their *M. tb* and *H. pylori* testing results, there was a relatively similar distribution of gender and ages among the four groups; however, the healthy controls were somewhat younger and more likely to be female (Table 1). The majority of the participants, 66 (82.5%), were men. As per the enrollment criteria, all participants were determined to be HIV seronegative.

Sensitivity and specificity of oral UBT for Pulmonary TB and *H. pylori*

Among TB mono-infected patients (Group 2, TB+/HP−) the highest oral UBT signal was detected at the 20-minute time point (data not shown); however, the signal levels were similar over the course of the three time points tested (10, 20 and 45 minutes) (data not shown). Hence we used the 20 minute time point to determine UBT positivity or negativity, and patients with DOB_{20} 2.4 were considered to be UBT positive.

Of the 80 patients enrolled in the study 14 (17.5%) were TB+/HP+ (Group 1), 20 (25%) were TB+/HP− (Group 2), 10 (12.5%) were TB−/HP+ (Group 3), and 36 (45%) were TB−/HP− (Group 4). More clinically relevant was that 32 of 36 (89%) TB−/HP− (Group 4) patients had significant urease activity with DOB_{20} 2.4. Also 14 of 20 (70%) TB+/HP− (Group 2) patients had DOB_{20} 2.4 (Table 2).

Using DOB_{20} 2.4 as the breakpoint for UBT positivity, oral UBT was calculated to have a sensitivity of 70% (95% CI: 46–88%) to diagnose TB among *H. pylori* stool antigen negative patients (TB+/HP−, Group 2). Among *H. pylori* mono-infected patients (TB−/HP+, Group 3), oral UBT had a calculated sensitivity of 100% (95% CI: 69–100%) to diagnose *H. pylori*. Despite a high or relatively high sensitivity, the calculated specificity was surprisingly low for both groups, only 11% (95% CI: 3–26%) (Table 3). Both groups shared the same negative control group composed of participants who were not actively infected with either *M. tb* or *H. pylori* (TB−/HP−, Group 4) which is why the specificity was identical. Specificity for *H. pylori* was far below the reported specificity of 96% using BreathTek UBT, suggesting that background urease contribution to UBT could be high in the Malian population.

Stool Microbiome Assessment

Given the low calculated specificity of oral UBT for both *H. pylori* and *M. tb* in this patient population, we analyzed DNA isolated from stool to assess for the presence of other microbes that might be contributing to a positive oral UBT test. Analysis of whole genome shotgun metagenomic data was performed on 10 healthy control participants with no clinical or laboratory evidence of either *M. tb* or *H. pylori* infection (TB−/HP−, Group 4) but with a positive oral-UBT. Neither *M. tb* nor *H. pylori* DNA was detected in any sample confirming the absence of *M. tb* and *H. pylori* infection in these patients as measured by clinical and laboratory testing. Thus, the negative sputum cultures and stool antigen results for mycobacteria and *H. pylori*, respectively, were not due to false negative test results. Of note, the sequencing did reveal the presence of multiple non-*H. pylori* ureases including bacterial encoded enzymes, *ureC*, *ureB*, *ureA*, and *ureAB* and invertebrate encoded enzymes, *ure1/ ure2*. When compared to 136 stool samples from healthy, U.S. volunteers who participated in the Human Microbiome Project (HMP), the relative abundance of urease genes was markedly higher among the Malian samples (Figure 2). This increased relative abundance of urease genes in the Malian samples was notable for both total numbers of urease genes and for all subsets of urease genes. Of the 279 distinct ureases detected across all samples, nearly 20% of reads aligning to ureases were from 10 bacterial genera including *Helicobacter* (Table 4). The *Helicobacter*-related ureases we identified in these stool samples matched ureases from the genomes of *H. hepaticus*, *H. mustelae*, *H. acinonychis* and *H. pylori*. Though we confirmed the absence of *H. pylori* based on the lack of other key *H. pylori* genomic signatures in these samples using a whole genome-based alignment approach, these data indicate that other species, likely including close relatives of *H. pylori*, are present and possibly contributing to positive UBT results among healthy Malians, and low specificity for *H. pylori* and *M. tb*.

DISCUSSION

In this study, we assessed the sensitivity and specificity of oral UBT as a diagnostic test for pulmonary TB in both *H. pylori* infected and uninfected populations in Mali. Our findings demonstrate only a moderate sensitivity of oral UBT (70%) for the diagnosis of active pulmonary TB among AFB-positive patients. As expected, oral UBT was 100% sensitive for the diagnosis of *H. pylori* infection. Surprisingly, our study revealed a remarkably low specificity of the UBT for the diagnosis of both TB and *H. pylori* (11%).

While the poor specificity for pulmonary TB could be partially explained by co-infections with *H. pylori*, the 11% specificity for *H. pylori* is far below the reported specificity of 96% (BreathTek). This could either be due to a higher degree of false positives in our study or a true difference between the patient populations utilized to determine the specificity of BreathTek compared to the Malian population in our study. The clinical trials used to evaluate the specificity and sensitivity of BreathTek were carried out mostly in developed countries. Those populations differ greatly from our patient sample in Mali with regards to diet, general health, genetics, and co-infections. Most importantly, *H. pylori* infection is endemic in Mali, which is not the case in developed countries. Consequently, the clinical trial results of BreathTek may not be representative of what we find in Mali. In addition to

this, it has been recently reported that the stool antigen test is a poor diagnostic test for the detection of *H. pylori* in Mali, which was not known when this study was conducted. In that report, Austarheim et *al.* found a sensitivity of 21% only for *H. pylori* stool antigen test in Mali in patients with gastric ulcer, although previous studies had reported 95% prevalence of *H. pylori* infection in Mali [21]. This lack of an appropriate gold standard for the diagnosis of *H. pylori* in Mali is therefore a limitation of this study.

A further explanation for the low specificity is that the presence of other organisms gave rise to a non-specific urease signal. To explore this hypothesis, we carried out a stool microbiome analysis of individuals who lacked either clinical or laboratory evidence of either *M. tb* or *H. pylori* infection. Stool whole genome shotgun sequencing did confirm that DNA from neither *M. tb* nor *H. pylori* was present among these samples. Instead, sequencing revealed that many urease-producing organisms other than *H. pylori* or *M. tb* were present in the stool, supporting our hypothesis. Additionally, this study revealed that asymptomatic participants from Bamako had at least three times more urease genetic material than those recruited for the Human Microbiome Project (HMP), further supporting our suspicion of false positivity due to other urease producers. However, we should note the limited number and lack of controls for this microbiome analysis as only 10 samples from TB−/HP− (Group 4) participants were tested.

Oral administration of urea for the detection of pulmonary TB may not be ideal for several reasons. First, after oral administration the concentrations of ^{13}C-urea reaching the lung are lower than those in the stomach and hence the amount of tracer available for bacterial conversion in the lung is limited. ^{13}C-urea uptake occurs rapidly once the tracer enters the jejunum, and earlier studies suggest that serum C_{max} concentrations are achieved by 30–45 minutes after ingestion [26, 27]. Although our previous study in the rabbit model of TB produced a positive signal by both intra-tracheal administration [18] and oral administration (unpublished data), the anatomical and physiological parameters of oral administration may render it a poor model for studies such as this. Second, the results are further confounded by the presence of *H. pylori* in the gut, particularly in an area where *H. pylori* is also endemic. We also considered the possibility of occult gastrointestinal tuberculosis as a potential confounder as an explanation for the high level of UBT positivity in TB−/HP− (Group 4) subjects; however, we consider this unlikely because gastrointestinal TB is rare, none of our subjects reported abdominal pain, night sweats, or fevers and our stool microbiota analysis of ten TB−/HP− (Group 4) subjects failed to identify *M. tb*. DNA.

In light of our findings of low specificity of oral UBT for identifying TB patients, we believe that alternative routes of ^{13}C-urea administration such as the intravenous or inhalation routes may circumvent issues of non-specificity due to gut urease producers. In this regard we note that a phase-1 trial of inhaled ^{13}C-urea as a pneumonia breath test is currently underway (NCT01303068) and that such a test may be more likely to render the encouraging results that were seen in *M. tb*.-infected animals receiving direct administration of ^{13}C-urea into the lungs [18]. Additionally, there may be a potential role of UBT either by the oral or breath route as a biomarker of TB therapy response, although further trials addressing this question are necessary.

In summary, our data suggest that oral UBT has significant limitations as a point of care diagnostic tool for pulmonary TB in a setting with endemic *H. pylori* infection. Aerosolized UBT, however, may hold potential for detection of TB or monitoring patients on antitubercular therapy. Oral UBT also had a poor specificity for the diagnosis of *H. pylori* in this setting, which may be due to the presence of non-*H. pylori* ureases that we detected by stool microbiome analysis. Beyond confounding of oral UBT testing results, the clinical significance of these non-*H. pylori* ureases remains to be determined.

Acknowledgments

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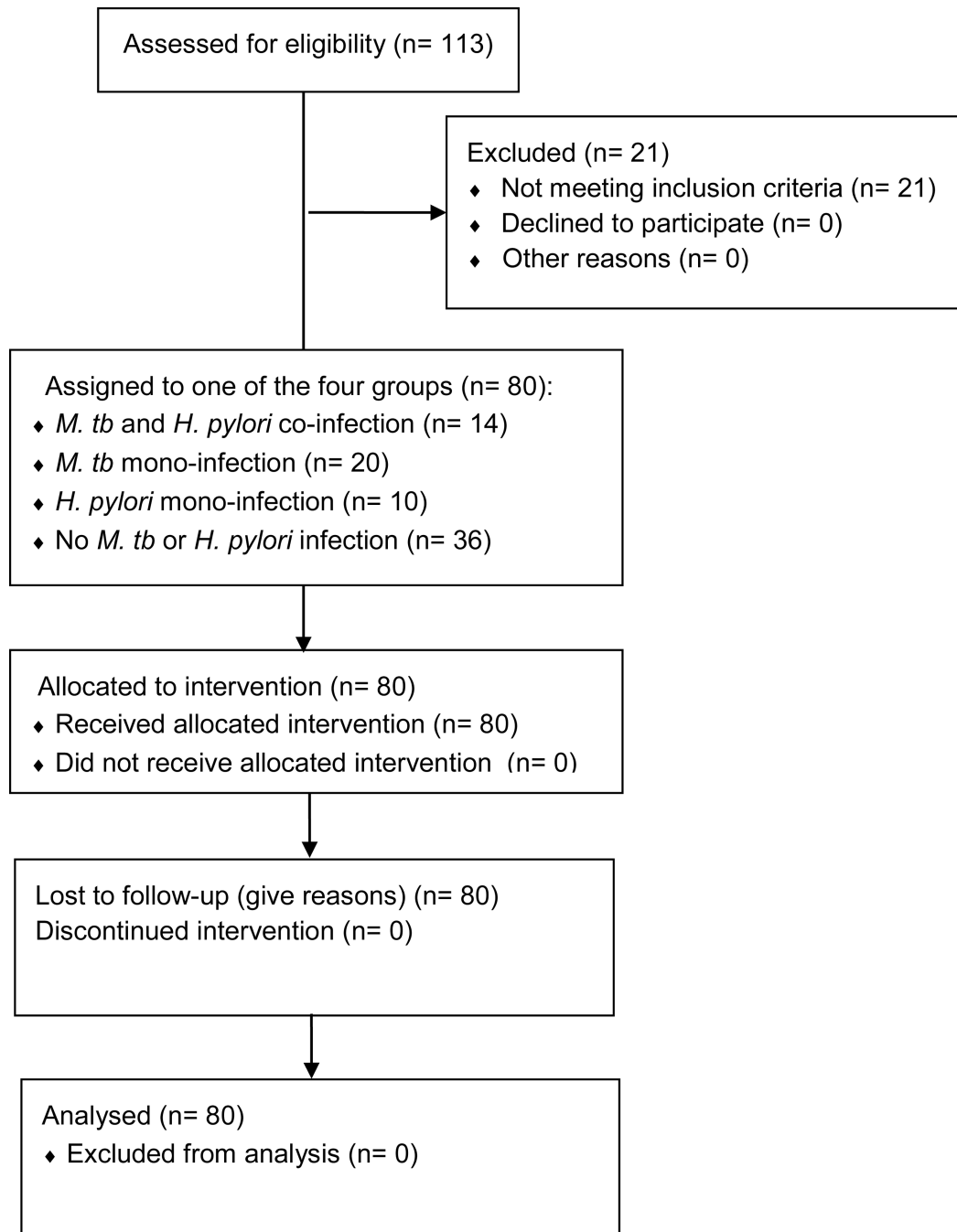
Figure 1.
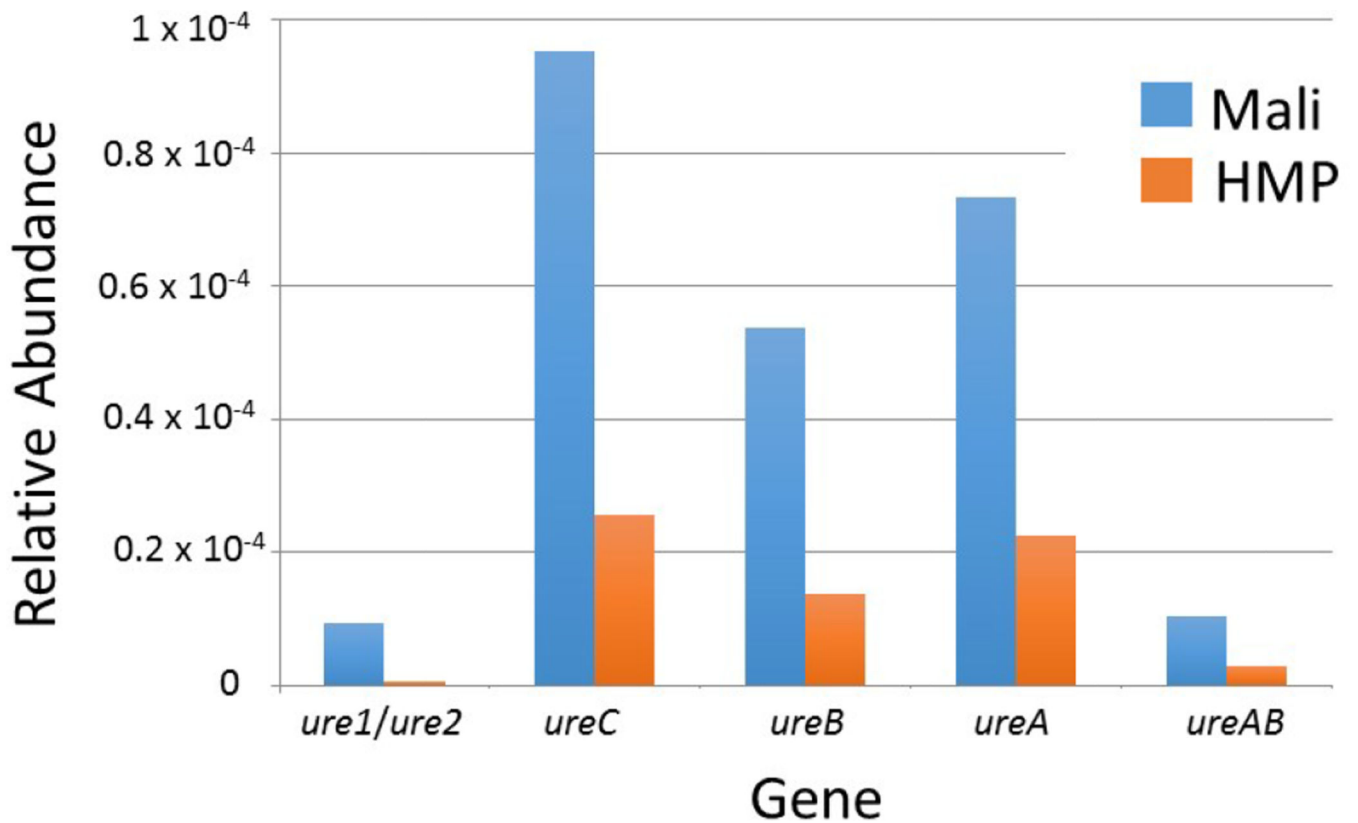CONSORT Flow Diagram shows study's groups and number of participants.

Figure 2.
Stool microbiome analysis of uninfected Malians (without *M. tb* or *H. pylori* infection but with positive oral UBT) revealed elevated levels of non-*Helicobacter pylori* ureases. The median relative abundances of urease gene content in the Malian samples versus those from 136 stool samples of healthy volunteers enrolled in the Human Microbiome Project (HMP) are displayed. When the relative abundance of ureases in the Malian data was compared to the HMP samples, the following fold-increases were noted: *ure1* and *ure2*: 19.1x; *ureC*: 3.7x, *ureB*: 3.8x, *urea:* 3.3x, *ureAB*: 3.4.

Table 1

Demographic characteristics at time of enrollment

	Group 1: *M. tb* and *H. pylori* co-infection (n = 14)	Group 2: *M. tb* mono-infection (n = 20)	Group 3: *H. pylori* mono-infection (n = 10)	Group 4: No *M. tb* or *H. pylori* infection (n = 36)
Male, n (%)	13 (93)	18 (90)	9 (90)	26 (72)
Mean age, yr (range)	36 (23–63)	29 (19–51)	30 (19–50)	28 (20–70)

Table 2

Group definitions and urea breath test (UBT) results using the delta-over-baseline at 20 minutes (DOB_{20}) 2.4 as the breakpoint for UBT positivity.

Patients	Infection	$DOB_{20} < 2.4$ (% within group)	DOB_{20} 2.4 (% within group)
Group 1	TB+/HP+	0	14 (100%)
Group 2	TB+/HP−	6 (30%)	14 (70%)
Group 3	TB−/HP+	0	10 (100%)
Group 4	TB−/HP−	4 (11%)	32 (89%)

Table 3

Oral UBT sensitivity and specificity for *M. tb* and *H. pylori* detection in comparison to mycobacterial culture or *H. pylori* stool antigen. Oral UBT was considered positive if signal 20 minutes following test initiation was greater than or equal to 2.4 fold delta-over-baseline ($DOB_{20} \geq 2.4$).

	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
M. tuberculosis	14	32	6	4	0.70 (0.46–0.88)	0.11 (0.03–0.26)
H. pylori	10	32	0	4	1.00 (0.69–1.00)	0.11 (0.03–0.26)

TP: true positive; FP: false positive; FN: false negative; TN: true negative.

Table 4

Top 10 bacterial genera for which urease genes were identified among stool samples of *M. tb* culture-negative and *H. pylori* stool-antigen negative Malians who were nevertheless positive for the oral UBT. A total of 279 distinct ureases were identified in these stool samples. The table shows the rank order of bacterial genera that contributed the highest average relative abundance of urease genes among all detected ureases.

Rank	Genus	Relative Abundance (Percent ± SD)
1	*Bacillus*	3.56 ± 0.34
2	*Helicobacter*	2.22 ± 0.49
3	*Synechococcus*	2.14 ± 0.13
4	*Corynebacterium*	1.92 ± 0.31
5	*Clostridium*	1.90 ± 0.37
6	*Prochlorococcus*	1.84 ± 0.04
7	*Pseudomonas*	1.74 ± 0.09
8	*Paenibacillus*	1.52 ± 0.14
9	*Staphylococcus*	1.43 ± 0.28
10	*Cyanothece*	1.34 ± 0.08