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GIARDIA DUODENALIS GLUTAMATE DEHYDROGENASE AND TRIOSE-PHOSPHATE ISOMERASE PCR-RFLP GENOTYPING EFFICIENCY AND PARASITE DENSITY

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ABSTRACT

Background. **Giardia duodenalis is an intestinal protozoan parasite, with human infections predominantly caused by assemblages A and B. Genotyping of G. duodenalis infections commonly relies on GDH and TPI gene targets using PCR-RFLP and sequencing approaches, which are widely applied across diverse epidemiological settings. However, the performance and discriminatory power of these molecular tools can vary depending on parasite density, DNA quality, and the infecting assemblage, with assemblage B often demonstrating higher genetic heterogeneity. As such this study evaluates the effectiveness of GDH- and TPI-PCR-RFLP in characterizing G. duodenalis in relation to parasite density in patients with giardiasis at Busia county referral hospital, Kenya.**

Methodology **This hospital-based cross-sectional study was done at Busia County Referral Hospital from 2017 to 2020. A total of 147 patients referred to the clinical laboratory for stool analysis were recruited into the study. Genomic DNA was isolated from stool samples of 88 patients who tested positive for G. duodenalis by microscopic examination. The isolates were genotyped at the GDH and TPI gene loci using a semi-nested PCR-RFLP technique. Genotyping agreement between the GDH and TPI gene loci was analysed using Cohen's kappa statistics. Whereas parasite density were compared across the assemblages and sub-assemblages by Kruskal wallis and post hot dunnes analysis.**

Results. **This study showed that GDH and TPI single locus genotyping demonstrated no agreement in overall DNA amplification (Cohen's kappa, 0.125; P = 0.228) and assemblage (Cohen's kappa, 0.024; P = 0.234) genotyping success. Nevertheless, moderate or lack of agreement between GDH and TPI was detected for assemblage (Cohen's kappa, 0.435; P < 0.001) and sub-assemblage (Cohen's kappa, 0.027; P = 0.117) genotypes, respectively. The parasite density was marginally significantly higher in GDH (P = 0.067) but significantly lower in TPI (P = 0.032) in amplified cases. Furthermore, the parasite densities were significantly different amongst the assemblages (P = 0.014) and sub-assemblages (P = 0.003). Post-hoc analyses indicated a significantly higher parasite density in assemblage B relative to assemblage A (P < 0.001); BIII sub-assemblage compared to AI (P < 0.001), AIII (P < 0.001), and mixed AI/AIII (P < 0.001) sub-assemblages; as well as BIV versus AI (P < 0.001) sub-assemblages.**

Conclusions. **The findings show the influence of parasite density and genetic heterogeneity on genotyping efficiency and support the use of multi-locus approaches for more reliable characterisation of G. duodenalis infections.**

INTRODUCTION

Giardia duodenalis infection is widely prevalent in developing countries, contributing to a substantial poor health and economic burden on human and animal populations [1]. The high burden of disease is primarily due to limitations in current detection methods, leading to underdiagnosis and poor identification of infected individuals and transmission sources [2,3]. While microscopic stool examination is the cornerstone of giardiasis diagnosis in patients, the utility of this method in large epidemiologic surveillance studies is limited by low sensitivity [4]. Molecular methods based on sequence genome analyses demonstrating high sensitivity and specificity of detecting G. duodenalis have been applied in epidemiologic and clinical detection of giardiasis [5]. A majority of these techniques utilise DNA or RNA amplification followed by detection through enzymatic restriction fragment length polymorphism (RFLP), blotting, spectroscopy, immunoassays, and sequencing, among others [6,7]. Several G. duodenalis genes such as glutamate dehydrogenase (GDH), triose-phosphate isomerase gene (TPI), β-giardin (BG), small subunit ribosomal ribonucleic acid, elongation factor-1α, NADP-dependent malic enzyme, and variant-specific surface protein (VSPs) have been previously used in detecting the presence of G. duodenalis infections, including assemblages and sub-assemblages [6,8]. However, inconsistencies in detection efficiency and poor agreement across studies may hinder the development of standardised and effective intervention strategies in regions with similar transmission dynamics and risk profiles.

The efficiency of these molecular loci-specific detection methods varies greatly with the BG, GDH and TPI gene loci of the parasite infections and genotypes [9,10]. For instance, previous G. duodenalis genotyping of GDH and TPI genes in Egyptian children showed amplification success of 100% and 96.5%, respectively [9], while studies amongst Brazilian children illustrated success rates of 75.6% and 71.1%, respectively, for TPI and GDH genes [11]. Besides, a lower genotyping efficiency of

success at both TPI (65.6%) and GDH (76%) was reported in previous studies for G. duodenalis isolates from children residing in informal settlements in Nairobi, Kenya [12]. The underlying reasons for variability in genotyping efficiency using these genes are influenced by factors such as primer design, and inherent properties and quantity of DNA templates [9,13]. To improve genotyping success, the multi-locus approach of at least two genes is applied in most clinical-epidemiological studies [14]. For instance, higher parasite DNA levels were associated with greater genotyping success for assemblage B G. duodenalis infections in giardiasis patients from Bangladesh [15]. Likewise, studies in Brazil amongst children demonstrated greater parasite cyst levels in assemblage B, including sub-assemblage BIII and BIV parasites [16], further suggesting that genotyping success, is governed by the strains of the predominant G. duodenalis infection. Therefore, the study evaluated the agreement of GDH and TPI PCR-RFLP genotyping in relation to parasite density in giardiasis patients at Busia County referral hospital, Kenya.

MATERIAL AND METHODS

Study setting and methods. Hospital-based cross-sectional study was carried out at Busia County Referral Hospital from 2017 to 2020. A total of 147 patients referred to the clinical laboratory for stool analysis were recruited into the study. The study design and methods are described in our recent publication [17]. A total of 88 microscopy-confirmed G. duodenalis–positive stool samples were collected from outpatients aged 3–73 years at Busia County Referral Hospital, western Kenya. The samples were obtained after informed consent or assent and were used for DNA extraction. DNA extraction was done using QiAmp® DNA stool Mini kit (Qiagen, UK) as per the manufacturer's methods, with an additional 5-minute vortexing of stool-glass bead mixture [18]. The parasite genome DNA was then genotyped independently by (RFLP) using primers at (GDH) and (TPI) gene loci as previously described [6]. In summary, Semi-nested PCR (nPCR) amplified the GDH gene locus HQ616623 exon IV, generating a ~432 bp fragment using primers: '5-TCAACGTYAAYCGYGGYTTCCGT-3' for primary reaction; and 5'-GTTRTCCTTGCACATCTCC-3', and 5'-CAGTACAACTCYGCTCTCGG-3' for secondary amplification as previously demonstrated [19]. While at TPI gene loci HQ179643 on exon III was amplified using primer '5-AAATIATGCCTGCTCGTCG-3' and '5-CAAACCTTITCCGCAAACC-3' for the first PCR reaction into a 605-bp template, it was further amplified using primers '5-CCCTTCATCGGIGGTAACTT-3' and '5-GTGGCCACCACICCCGTGCC-3' for the second PCR reaction for a 532-bp product. The primary and the secondary reactions were performed under the following conditions; 1cycle of 94°C for 2min, 56°C for 1 minute and 72°C for 2 minutes, followed by 55 cycles of 94°C for 30s, 56°C for 20s and 72°C for 45s, and a final extension of 72°C for 7 minutes, as previously described [20].

The reaction was performed in a 25 µl volume as previously described (Read et al., 2004). The amplified products of the GDH gene loci were digested using two restriction enzymes: NIa IV (New England Biolabs, USA) for the (532 bp fragment) and RSaI (New England Biolabs, USA) for (605 bp fragment) for assemblage and sub-assemblage discrimination [19]. The amplicons for the TPI gene loci were digested with 10 units of endonucleases BbvI, RsaI and MnlI (New England Biolabs Inc., USA) for (432 bp fragment) for assemblage and sub-

assemblage differentiation [20]. The digested products were resolved by electrophoresis (sub-cell model 192 electrophoresis systems, Bio-Rad, USA) at 100 volts for 45 minutes at room temperature in 2% agarose gels (Invitrogen, USA) stained with 0.5 mg/ml ethidium bromide. The resolved fragments were visualised under UV light using the gel documentation system (Uvitec, UK) and compared with the band size against positive internal controls (molecular ladder).

Data management. Data analysis was conducted using the statistical package for social sciences (IBM® SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp., USA). Agreement between the GDH and TPI loci in genotyping for interrater reliability was done using Cohen's kappa statistic [21]. Parasite densities were compared between successfully and unsuccessfully genotyped GDH and TPI loci using the Mann-Whitney U test. In addition, parasite densities were compared amongst the assemblages and sub-assemblages using the Kruskal-Wallis test, followed by post-hoc correction by Dunn's test.

Ethical approvals. Ethical approvals of the study were obtained from the institution's scientific and ethics review committee of the Masinde Muliro University of Science and Technology (MU/403012-V36), the National Commission for Science, Technology, and Innovation (NACOSTI:436602).

RESULTS

Agreement between GDH and TPI loci genotyping.

Comparison of the GDH and TPI single locus genotyping success (Table 1) showed no agreement in the success of parasite DNA amplification for all cases (Cohen's kappa, 0.125; coefficient of determination, 0.37%; P = 0.228) and assemblages (Cohen's kappa, 0.024; coefficient of determination, 0.41%; P = 0.234) yield. However, a moderate and lack of agreement between GDH and TPI was noted for sub-assemblages (Cohen's kappa, 0.435; coefficient of determination, 23.04%; P < 0.001) and mixed assemblages (Cohen's kappa, 0.027; coefficient of determination, 0.05%; P = 0.117) genotyping.

Table 1

Agreement between GDH and TPI reliability in Giardia duodenalis genotyping

Variables	GDH, n (%)	TPI, n (%)	Kappa	SE kappa	COD (%)	P
Cases	64 (72.7)	75 (85.2)	0.125	0.061	0.37	0.228
Assemblages						
A	15 (23.4)	16 (21.3)				
B	44 (68.8)	46 (61.4)	0.024	0.064	0.41	0.234
A/B	5 (7.8)	13 (17.3)				
Sub-assemblages						
AI	5 (7.8)	4 (5.3)				
AII	7 (10.9)	6 (8.0)				
AIII	2 (3.1)	3 (4.0)	0.435	0.048	23.04	<0.001
BIII	27 (42.2)	18 (24.0)				
BIV	14 (21.9)	14 (18.7)				
Mixed sub-assemblages						
AI/AII	1 (1.6)	1 (1.3)				
AI/AIII	0 (0.0)	2 (2.7)	0.027	0.023	0.05	0.117
AII/BIII	5 (7.8)	13 (17.3)				

BIII/BIV	3 (4.7)	14 (18.7)

Results are presented as numbers (n) and proportion of successful glutamate dehydrogenase (GDH, n=64) and triose phosphate isomerase (TPI, n=75) loci genotyping. Analysis for performance agreement of genotyping for interrater reliability was based on Cohen's kappa; SE of kappa, standard error, and COD, coefficient of determination [21]. *P-values in bold are statistically significant.*

Comparison of Giardia duodenalis parasite densities in assemblages and sub-assemblages. Enumeration of trophozoites and/or cyst shedding in successfully genotyped cases at each locus (Table 2) indicated that the parasite density was marginally significantly higher in GDH (P = 0.067) but significantly lower in TPI (P = 0.032) amplified cases.

Table 2

Comparison of parasite density in GDH and TPI genotyped cases

Parasite density	GDH			TPI		
	No	Yes	P	No	Yes	P
Trophozoites/µl stool, median (IQR)	5.9 (1.0)	7.1 (4.0)	0.067	9.8 (7.0)	6.34 (3.0)	0.032

Giardia duodenalis parasite density was expressed as median (interquartile range, IQR) of trophozoites and/or cyst/µl of stool. The P-value in bold was statistically significant.

Differences in Giardia duodenalis parasite densities in assemblages and sub-assemblages.
A comparison of parasite densities was significantly different across assemblages (Figure 1a; P = P=0.028) and sub-assemblages (Figure 1b; P = 0.004). Post-hoc analyses revealed that the parasite density was significantly higher in carriers with assemblage B infections relative to assemblage A infection carriers (P = 0.006). Likewise, the parasite density was significantly higher in the BIII sub-assemblage relative to AII (P = 0.042) and mixed (P = 0.003) sub-assemblages.

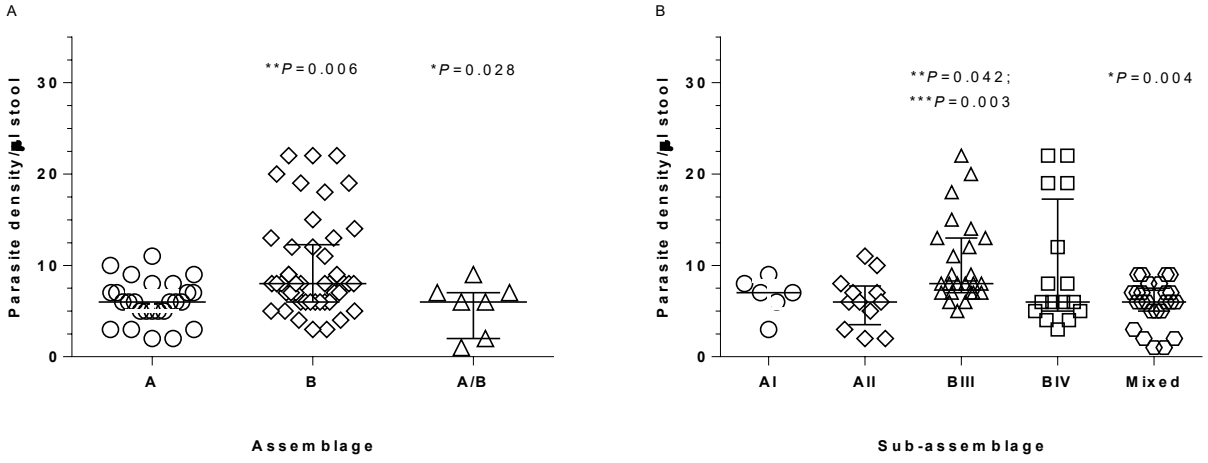

Figure 1 shows scatter box-plots of median (interquartile range, IQR) parasite (trophozoites and/or cysts) density/µl of stool in Giardia duodenalis assemblage (Figure 1A) and sub-assemblage (Figure 1B) infections.

Figure 1A shows scatter box-plots of median parasite (trophozoites and/or cysts) density/µl of stool with the circle, rhombus, and triangles representing assemblages A, B, and mixed assemblages, respectively. Figure 1B shows scatter box-plots of the median parasite (trophozoites and/or cysts) density/µl of stool with the circle, rhombus, triangle, square, and hexagon representing sub-assemblage AI, AII, BIII, and BIV, and mixed sub-assemblage AI/AII, AI/AIII, BIII/BIV and AII/BIII, respectively. Kruskal-Wallis test (*P) with post-hoc Dunn's test (**P and ***P) were used to examine differences in trophozoite and/or cyst counts across the assemblages and sub-assemblages. *P = 0.028 and *P = 0.004 across assemblages and sub-assemblages, respectively. **P = 0.006 vs. assemblage A; **P = 0.042 vs. sub-assemblage AII; and ***P vs. mixed sub-assemblages.

DISCUSSION

In the current study, we examined the agreement between GDH and TPI single locus genotyping efficiency of genome DNA extracted from stool specimens stored in 2.5% potassium dichromate for one year at -40^{o}C. The results show no agreement in genotyping success at both GDH and TPI loci, for overall amplification yield, assemblages and mixed sub-assemblages. However, a moderate agreement was observed in the detection of sub-assemblages. These observations, in part, are attributable to differences in the base sequence at the two gene loci. This proposition aligns with the fact that most molecular techniques for detecting and genotyping Giardia duodenalis rely on amplifying gene fragments using oligonucleotides that target conserved DNA sequences. These methods include nested PCR followed by RFLP, gene or whole-genome sequencing, cloning, real-time PCR, or high-resolution melting analysis [22,23]. Currently, epidemiological investigations use multi-locus typing assays to distinguish between environmental, anthroponotic, and anthropozoonotic sources of infection and to identify mixed infections. These assays also enhance the detection of genetic exchanges between parasite strains and link specific genotypes to disease presentations [6,24].

The greater amplification efficacy at higher and lower parasite densities for GDH and TPI loci, respectively, can be explained by differences in hybridisation and amplification efficiencies at the two loci due to the ability to pick the polymorphisms at higher and lower DNA quantities, respectively. This result is consistent with previous studies indicating higher sensitivity for TPI genotyping at a lower trophozoite threshold of 20 parasites/µl [25]. However, interference by the host and other intestinal microbial factors, such as complex polysaccharides and lipids, can differentially affect the efficiency of G. duodenalis gene amplification in the two genes [26]. Likewise, the difference may also be related to lower GC content in both gene loci, the larger fragment sizes of TPI (605 and 532bp) compared to GDH (432bp), and the higher potential base-pair dimerisation sites in the GDH (20) relative to TPI (15) fragments. These findings align with previous G. duodenalis genotyping characterisation, demonstrating that the TPI gene locus is GC-rich [12]. Additional results illustrating higher parasite density in assemblage B and sub-assemblage BIII and BIV relative to assemblage A, and sub-assemblage AI and AII genotypes, suggest more DNA yield for the B assemblage parasites. This finding is consistent with previous studies in Bangladesh amongst giardiasis patients showing higher parasite

DNA loads in assemblage B parasites [27]. Besides, studies in Brazil amongst children demonstrated greater parasite cyst levels in assemblage B, including sub-assemblage BIII and BIV parasites [28]. Altogether, we can deduce that genotyping efficiency at the two gene loci depends on the parasite density and inherent characteristics of primers used in the genotyping assays. This study applied the widely used multi-locus genotyping strategy based on the constitutively expressed genetic markers GDH and TPI loci in genotyping for G. duodenalis assemblages and sub-assemblages amongst patients from a rural setting with a high burden of giardiasis [29].

Study limitation. In the current study, we analyse GDH and TPI gene loci; there is a need for additional genes, such as the small subunit ribosomal RNA and the beta-giardin genes can be incorporated into multi-locus approaches for further confirmation of the assemblages and sub-assemblages [30]. In addition, only one sample was collected from each patient and stored in 2.5% potassium dichromate for one year before batched analysis. It should be noted that the shedding of trophozoites and cysts is intermittent, usually occurring at peak parasite density at about one-week intervals, requiring the collection of multiple stool specimens for successful DNA yield [31].

CONCLUSION

This study demonstrates limited concordance between GDH and TPI single-locus PCR-RFLP genotyping of Giardia duodenalis, with poor agreement in overall DNA amplification and assemblage assignment, underscoring locus-dependent variability in genotyping performance. While GDH and TPI showed moderate agreement at the assemblage level, agreement at the sub-assemblage level was minimal, highlighting challenges in resolving finer genetic diversity using single loci. Parasite density significantly influenced genotyping outcomes, with differential effects observed between GDH and TPI amplification, and marked variation across assemblages and sub-assemblages. Notably, assemblage B and its sub-assemblages, particularly BIII and BIV, were associated with significantly higher parasite densities compared to assemblage A and related sub-assemblages. Overall, these findings emphasise the influence of parasite density and genetic heterogeneity on genotyping efficiency and support the use of multi-locus approaches for more reliable characterisation of G. duodenalis in a rural setting with high burden of giardiasis.

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