Glutamate dehydrogenase (GDH) is Highly Conserved Among *Clostridium difficile* Ribotypes

K.N. Gearhart*, R.J. Carman, L. Chen, A.M. Lawrence, J.H. Boone, D.M. Lyerly

TechLab®, Inc., Blacksburg, VA 24060-6358, USA

Background:

Immunoassays for GDH, a metabolic enzyme encoded by *gluD*, are used to detect *C. difficile* in diarrheic fecal samples. The diagnostic utility of GDH requires isolates of all ribotypes to react in such assays.

Recent reports (Tenover et al., 2010; Carroll, 2011) claim commercial GDH assays miss some ribotypes, in particular ARL 002, ARL 027, and ARL 106. It has been proposed that these ribotype-specific false negative results can be explained by *gluD* negative ribotypes and/or antigenic variation in GDH.

Aims:

1) To amplify the *gluD* gene of 84 *C. difficile* ribotypes by PCR and screen immunoreactivity in GDH immunoassays.
2) To quantify *in vitro* GDH production of all 88 ribotypes.
3) To sequence $gluD_{65\text{-}1218}$ and compare predicted amino acid sequences.

Materials and methods

***C.difficile* Isolates:** 84 ribotypes (including ARL 001, 002, 003, 012, 014, 017, 027, 033, 036, 043, 053, 054, 078, 106, 110, 126, and 154) were subcultured to 5mL Brain Heart Infusion (BHI) broth tubes (Anaerobe Systems, Morgan Hill, CA) and incubated anaerobically at 37ºC for 72 hours.

GDH Assays: 72 hour BHI broth cultures were tested in the *C. DIFF QUIK CHEK®*, *C. DIFF QUIK CHEK COMPLETE®*, and the *C.DIFF CHEK™-60* tests (TechLab®, Inc., Blacksburg, VA).

Bacterial DNA: DNA was extracted using the QIAGEN DNA Mini Kit (Valencia, CA). Eluted DNA was stored at <-10ºC until ready for analysis.

PCR: *gluD*, encompassing the glutamine and NAD binding domains, from 27 ribotypes was amplified by PCR. See Table 1.

Sequencing: PCR products were sequenced by VBI (Blacksburg, VA) and then analyzed using ClustalW2 (EMBL-EBI).

Toxins and GDH Immunoassay: A quantitative immunoassay was used to detect the amount of GDH. Broth cultures were diluted, centrifuged, and filtered. The filtrate was incubated for 30 mins with specific capture antibodies. A second 30 min incubation with detection antibodies was done. A fluorescence detection was performed prior to analysis.

TABLE 1: PCR conditions and primers for *gluD* amplification

Gene amplified	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Thermocycling Conditions
gluD	ATGTCAGGAAAAG ATGTAAATGTCTT CGAG	TTAGTACCATCCT CTTAATTTCATAG CTTC	35 cycles 94°C 30 sec 60°C 1 min 72°C 1 min

FIGURE 1: Representative electrophoresis gel for *gluD* amplicons

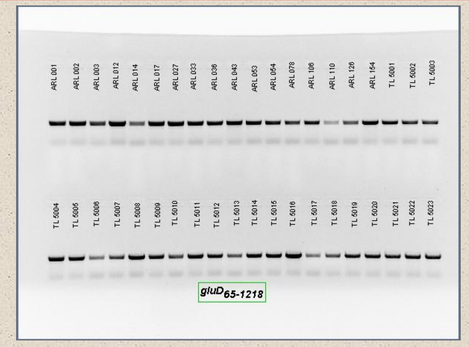
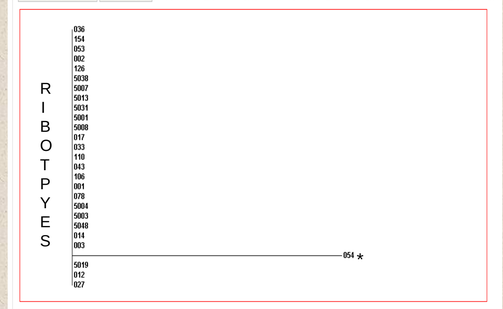


FIGURE 2: Phylogram for $gluD_{65\text{-}1218}$ predicted amino acid sequence alignment



*Sequencing showed 16 SNPs in $gluD_{65\text{-}1218}$ from the 27 most abundant ribotypes in our collection. Of these, only one led to a change in the predicted amino acid sequence. The predicted change, Valine to Leucine at 217aa in ARL 054, involved a conservative shift, from one non-polar amino acid with hydrophobic side chains to another. It was not associated with any changes in the immunoreactivity of GDH in ARL 054.

Ribotype	Strain	Toxin Phenotype	*C.DIFF QUIK CHEK®* (30390)	*C.DIFF QUIK CHEK COMPLETE®* (30550C) GDH only	*C.DIFF CHEK™-60* (30392)	In house quantitative assay (GDH ng/mL)
ARL 001	Pitt 02	A+/B+	+	+	3.922	5469.0
ARL 001	VPI 8491	A+/B+	+	+	4.183	9917.0
ARL 002	BAA-1874	A+/B+	+	+	4.340	11986.0
ARL 002	UVA 10	A+/B+	+	+	3.824	5166.0
ARL 003	VPI 10463	A+/B+	+	+	3.863	4632.0
ARL 012	VPI 630	A+/B+	+	+	3.883	2410.0
ARL 012	BAA-1382	A+/B+	+	+	4.256	8571.0
ARL 014	UVA 30	A+/B+	+	+	3.919	2921.0
ARL 014	RMA 15549	A+/B+	+	+	4.326	8384.0
ARL 017	F1470	A-/B+	+	+	3.844	3907.0
ARL 017	Pitt 89	A-/B+	+	+	3.947	>3125
ARL 027	Pitt 45	A+/B+	+	+	3.859	3414.0
ARL 027	CD 196	A+/B+	+	+	3.887	3026.0
ARL 033	Is58	A-/B-	+	+	3.807	8127.0
ARL 033	J6090	A-/B-	+	+	4.195	19633.0
ARL 036	8864	A-/B+	+	+	3.848	2213.0
ARL 043	NCTC 11382	A+/B+	+	+	4.099	7403.0
ARL 053	BAA-1873	A+/B+	+	+	4.370	12108.0
ARL 053	Bartlett 1136	A+/B+	+	+	4.127	9658.0
ARL 054	VPI 13071	A+/B+	+	+	4.137	12037.0
ARL 078	Pitt 07	A+/B+	+	+	3.900	3101.3
ARL 106	NCTC 13404	A+/B+	+	+	4.300	2054.3
ARL 110	J6097	A-/B+	+	+	4.388	13749.0
ARL 154	BAA-1872	A+/B+	+	+	4.267	6790.0
ARL 126	BAA-1875	A+/B+	+	+	4.326	22021.0
TL 5001	CTH 124	A+/B+	+	+	4.562	8414.0
TL 5002	CTH 112	A+/B+	+	+	4.211	13651.0
TL 5002	Bartlett 223	A+/B+	+	+	4.658	8944.0
TL 5003	Pitt 336	A+/B+	+	+	4.370	7437.0
TL 5004	Pitt 46	A+/B+	+	+	4.305	1141.0
TL 5004	CCL 19561	A+/B+	+	+	4.220	760.2
TL 5005	Pitt 49	A+/B+	+	+	4.640	4005.0
TL 5006	ID1 follow up	A+/B+	+	+	4.390	7235.0
TL 5006	CCL 19587	A+/B+	+	+	4.434	698.3
TL 5007	CCL 17021	A+/B+	+	+	4.583	12522.0
TL 5007	CTH 050	A+/B+	+	+	4.569	12066.0
TL 5008	CTH 205	A-/B-	+	+	4.701	6312.0
TL 5009	CCL 3559	A+/B+	+	+	4.419	1883.2
TL 5010	RMA 8907	A+/B+	+	+	4.504	6071.0
TL 5011	Pitt 154	A+/B+	+	+	4.393	5878.0
TL 5011	CCL 19873	A+/B+	+	+	4.093	16351.0
TL 5012	CCL 17624	A+/B+	+	+	4.398	14258.0
TL 5012	CCL 19829	A+/B+	+	+	4.481	1634.4
TL 5013	CCL 18339	A-/B-	+	+	4.498	10418.0
TL 5013	CCL 19033	A-/B-	+	+	4.292	>3125
TL 5014	CTH 136B	A-/B-	+	+	4.692	7057.0
TL 5015	Pitt 337	A+/B+	+	+	4.335	5571.0
TL 5015	CCL 989	A+/B+	+	+	4.313	10134.0
TL 5016	CCL 17636	A-/B-	+	+	4.376	3517.0
TL 5016	CCL 18962	A-/B-	+	+	4.090	228.6
TL 5017	CCL 14041	A-/B-	+	+	1.366	428.1
TL 5018	CTH 026	A+/B+	+	+	4.311	9546.0

Ribotype	Strain	Toxin Phenotype	*C.DIFF QUIK CHEK®* (30390)	*C.DIFF QUIK CHEK COMPLETE®* (30550C) GDH only	*C.DIFF CHEK™-60* (30392)	In house quantitative assay (GDH ng/mL)
TL 5019	VPI 26689	A+/B+	+	+	3.741	4156.0
TL 5020	CCL 19353	A+/B+	+	+	4.531	10056.0
TL 5021	VPI 11186	A-/B-	+	+	3.879	3225.0
TL 5022	CCL 19084	A+/B+	+	+	4.589	9053.0
TL 5023	RMA 15024	A+/B+	+	+	4.253	>312.5
TL 5023	CCL 18270	A+/B+	+	+	4.332	>3125
TL 5024	CCL 17785	A-/B-	+	+	4.499	3582.0
TL 5025	CCL 18241	A+/B+	+	+	4.410	>3125
TL 5026	CCL 1012	A+/B+	+	+	4.286	6810.0
TL 5027	UVA 39	A-/B-	+	+	4.387	1767.0
TL 5028	CCL 18043	A+/B+	+	+	4.487	8788.0
TL 5031	CCL 19917	A+/B+	+	+	4.353	15344.0
TL 5031	Bartlett 1139	A+/B+	+	+	4.380	9574.0
TL 5032	Pitt 51	A+/B+	+	+	3.900	3128.0
TL 5033	J6009	A-/B-	+	+	4.148	1914.0
TL 5034	J1413	A-/B-	+	+	4.612	16905.0
TL 5035	CCL 19172	A+/B+	+	+	4.184	1328.4
TL 5036	CCL 14066	A-/B-	+	+	4.209	9380.0
TL 5037	HMC 8271	A+/B+	+	+	4.543	2188.0
TL 5038	Bartlett 234	A-/B-	+	+	4.374	5329.0
TL 5039	GT 236	A-/B-	+	+	too high	2756.0
TL 5040	CCL 18057	A-/B-	+	+	4.268	907.9
TL 5041	RMA 10790	A-/B-	+	+	4.528	6400.0
TL 5042	CCL 1037	A+/B+	+	+	4.244	9071.0
TL 5043	CCL 17516	A+/B+	+	+	4.350	1903.1
TL 5044	Germany 11703	A+/B+	+	+	4.171	1710.7
TL 5045	CCL 17369	A-/B-	+	+	4.324	2375.0
TL 5046	CCL 17504	A-/B-	+	+	4.255	6846.0
TL 5047	Germany 23027	A-/B-	+	+	4.463	>3125
TL 5048	RMA 15187	A+/B+	+	+	4.421	3957.0
TL 5048	CCL 19929	A+/B+	+	+	4.300	15540.0
TL 5049	CCL 13762	A+/B+	+	+	4.336	3799.0
TL 5050	CCL 19305	A+/B+	+	+	4.252	225.3
TL 5051	Bartlett 1053	A+/B+	+	+	4.429	13724.0
TL 5052	CCL 19563	A-/B_	+	+	2.766	590.4
TL 5053	RMA 9396	A+/B+	+	+	4.260	8882.0
TL 5054	CCL 18355	A+/B+	+	+	4.405	3463
TL 5057	HMC 1236	A-/B-	+	+	4.236	2007.0
TL 5059	CCL 17597	A-/B-	+	+	4.242	13496.0
TL 5062	CCL 19519	A-/B-	+	+	4.026	1026.1
TL 5063	CCL 19010	A+/B+	+	+	too high	6174.0
TL 5064	CCL 19541	A-/B-	+	+	4.629	425.2
TL 5065	CCL 17364	A+/B+	+	+	4.384	6345.0
TL 5066	Pitt 251	A+/B+	+	+	4.168	>3125
TL 5067	CCL 17961	A+/B+	+	+	4.306	9964.0
TL 5069	CCL 19083	A+/B+	+	+	4.586	9112.0
TL 5070	CCL 19216	A+/B+	+	+	4.675	12618.0
TL 5072	CCL 19600	A+/B+	+	+	4.143	1073
TL 5073	CCL 19637	A+/B+	+	+	too high	3125.0
TL 5074	CCL 18426	A-/B-	+	+	4.316	570.3
TL 5075	CCL 18319	A+/B+	+	+	4.143	>3125
TL 5076	CCL 19897	A-/B-	+	+	4.166	>3125

Results:

1) *gluD* gene was amplified from all 84 ribotypes, including 002, 027 and 106; there were no apparent size differences between amplicons. All ribotypes were positive in the GDH immunoassays.
2) All ribotypes expressed readily detectable levels (>12.5 ng/mL) of GDH *in vitro*.
3) All 27 predicted amino acid sequences generated from $gluD_{65\text{-}1218}$ DNA sequences, were identical with the exception of ARL 054.

Conclusions:

Because all 104 isolates of 84 ribotypes assayed contain *gluD* gene, and each reacted in all three GDH immunoassays, our results suggest that *gluD* negative ribotypes do not exist nor cause ribotype-specific false negative results. All isolates made easily detectable levels of GDH *in vitro*. Furthermore, no variation in the size of the *gluD* amplicon nor the predicted amino acid sequence it generates was observed, suggesting that there is no antigenic variation in GDH.

References:

Carroll, KC. 2011. Testing for *Clostridium difficile* in the Clinical Microbiology Laboratory ASCP Webcast April 6th, 2011.

Tenover et al. 2010. Impact of Strain Type on Detection of Toxigenic *Clostridium difficile:* Comparison of Molecular Diagnostic and Enzyme Immunoassay Approaches. J. Clin. Microbiol., 48:3719-24.

*Please forward questions or comments to kgearhart@techlab.com