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Defining the Phylogenomics of *Shigella* Species: a Pathway to Diagnostics

Jason W. Sahl,[a,b,c] Carolyn R. Morris,[a] Jennifer Emberger,[a] Claire M. Fraser,[a] John Benjamin Ochieng,[d] Jane Juma,[d] Barry Fields,[e] Robert F. Breiman,[e] Matthew Gilmour,[f]* James P. Nataro,[g] David A. Rasko[a]

University of Maryland School of Medicine, Institute for Genome Sciences, Department of Microbiology and Immunology, Baltimore, Maryland, USA[a]; Translational Genomics Research Institute, Flagstaff, Arizona, USA[b]; Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, Arizona, USA[c]; KEMRI-CDC Kisumu, Kisumu, Kenya[d]; CDC Kenya, KEMRI Headquarters, Nairobi, Kenya[e]; National Microbiology, Public Health Agency of Canada, Winnipeg, Manitoba, Canada[f]; Department of Pediatrics, University of Virginia, Charlottesville, Virginia, USA[g]

Shigellae cause significant diarrheal disease and mortality in humans, as there are approximately 163 million episodes of shigellosis and 1.1 million deaths annually. While significant strides have been made in the understanding of the pathogenesis, few studies on the genomic content of the *Shigella* species have been completed. The goal of this study was to characterize the genomic diversity of *Shigella* species through sequencing of 55 isolates representing members of each of the four *Shigella* species: *S. flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae*. Phylogeny inferred from 336 available *Shigella* and *Escherichia coli* genomes defined exclusive clades of *Shigella*; conserved genomic markers that can identify each clade were then identified. PCR assays were developed for each clade-specific marker, which was combined with an amplicon for the conserved *Shigella* invasion antigen, IpaH3, into a multiplex PCR assay. This assay demonstrated high specificity, correctly identifying 218 of 221 presumptive *Shigella* isolates, and sensitivity, by not identifying any of 151 diverse *E. coli* isolates incorrectly as *Shigella*. This new phylogenomics-based PCR assay represents a valuable tool for rapid typing of uncharacterized *Shigella* isolates and provides a framework that can be utilized for the identification of novel genomic markers from genomic data.

Shigellae are intracellular Gram-negative pathogens that cause a wide range of illnesses, from mild abdominal discomfort to death, in humans and nonhuman primates (1). The estimated 165 million cases of shigellosis that occur annually (2) result in the deaths of ~1.1 million people, most in the developing world. An additional 500,000 cases of shigellosis occur in travelers from developed countries (3). In the recent landmark Global Enteric Multisite Study (GEMS), *Shigella* species were identified as being among of the pathogens most associated with mortality (4, 5). Additionally, the GEMS suggested that houseflies could contribute to the spread of *Shigella*, introducing a novel route of transmission of this human pathogen (6). There are four species of *Shigella* that can cause diarrheal disease in humans, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*, formally termed serotypes A to D (7). These species are currently defined by serotyping based on components of the O-specific side chain of the lipopolysaccharide.

Shigella species are frequently identified in the laboratory by their lack of both motility and lactose fermentation, but those biochemical assays often cannot differentiate *Shigella* from some enteroinvasive *Escherichia coli* (EIEC) isolates (8). Additionally, clinical symptoms often cannot differentiate *Shigella* infection from *Escherichia coli* infection or distinguish between *Shigella* species (8). Further confounding accurate identification, some O antigens present in *Shigella* are identical to those found in *E. coli* (9). Serotyping is the current gold standard method for *Shigella* species determination, but cross-reactivity among *Shigella* isolates, and *E. coli* isolates, may confound results (10).

The diversity of the isolates within the species, as well as the technical aspects of serotyping, make the identification of a molecular diagnostic an important objective for accurate study of the impact of this important human pathogen. In an attempt to circumvent the challenges associated with serotyping, several genetic methods, including ribotyping (11), restriction fragment length polymorphism (12), multilocus sequence typing (MLST) (13), multilocus variable-number tandem-repeat (VNTR) analysis (14), and multiplex PCR assays, have been explored to identify *Shigella* isolates. These approaches remain overly labor-intensive or are not sensitive enough and are unable to discriminate between *Shigella* species isolates or even certain *E. coli* isolates.

Previous MLST-based phylogenetic studies indicated that the *Shigella* species have emerged at least seven separate times from *E. coli* (15). However, MLST methods interrogate a relatively small number of conserved housekeeping genes (16). Furthermore, phylogenies inferred from concatenated MLST sequences have been demonstrated to not always be representative of phylogenies inferred using the entire conserved genomic core (17). Incongruities with gene-based trees from the sequences from a limited

Received 11 December 2014 **Returned for modification** 5 January 2015 **Accepted** 9 January 2015

Accepted manuscript posted online 14 January 2015

Citation Sahl JW, Morris CR, Emberger J, Fraser CM, Ochieng JB, Juma J, Fields B, Breiman RF, Gilmour M, Nataro JP, Rasko DA. 2015. Defining the phylogenomics of *Shigella* species: a pathway to diagnostics. J Clin Microbiol 53:951–960. doi:10.1128/JCM.03527-14.

Editor: N. A. Ledeboer

Address correspondence to David A. Rasko, drasko@som.umaryland.edu.

* Present address: Matthew Gilmour, Diagnostic Services Manitoba, Health Sciences Centre, Winnipeg, Manitoba, Canada.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.03527-14.

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doi:10.1128/JCM.03527-14

number of loci have been attributed to the high recombination rate observed in *E. coli* (18).

The genomic diversity of the *Shigella* species has not been studied in detail. This report contributes 55 draft *Shigella* assemblies to the community for the broadest analysis of *Shigella* species genomics to date. The goals of this study were to compare a significant number ($n = 69$) of *Shigella* genomes in order to (i) determine the phylogeny of a diverse set of *Shigella* isolates compared to sequenced *E. coli* and *Shigella* genomes using whole-genome sequence data, (ii) identify genomic differences between *Shigella* and *E. coli* genomes, and (iii) develop a molecular assay that identifies unknown *Shigella* isolates and classify them in a phylogenetic context. The use of comparative genomics to identify and validate *Shigella*-specific phylogenetic markers has provided an opportunity to accurately and rapidly identify these important human pathogens. Additionally, this report provides a framework for the use of genomic data in the development of diagnostics for any species.

MATERIALS AND METHODS

Strain selection. A total of 55 *Shigella* genomes were selected from our culture collection for sequencing in an effort to capture a broad range of genomic, geographic, and temporal diversity (Table 1); 30 of these genomes were sequenced as part of the NIAID Genome Sequencing Center for Infectious Diseases (GSCID) project (http://gscid.igs.umaryland.edu/wp.php?wp = emerging _diarrheal_pathogens). Additional sequenced isolates included a collection of *Shigella* isolates ($n = 12$) from Nyanza Province, western Kenya, collected by the Kenya Medical Research Institute (KEMRI)/Centers for Disease Control and Prevention (CDC) Research and Public Health Collaboration that were associated with lethal clinical outcomes (19), a set of isolates from Chile ($n = 2$) used in previous studies (20, 21), and a collection of Canadian isolates ($n = 11$) obtained from the Public Health Agency of Canada through routine surveillance from 2008 to 2011; all *Shigella* isolates were identified based on serological analyses. A list of 281 additional genomes downloaded from GenBank, including completed genomes as well as draft assemblies, as well as the reads for an additional 96 *S. flexneri* genomes for comparative analyses are listed in Table S1 in the supplemental material.

DNA extraction. For genomic sequencing, DNA was extracted with standard methods reported previously (17). For the multiplex PCR assay, genomic DNA was prepped with a GenElute kit (SigmaAldrich). As a proof of concept, DNA was also isolated by heating 100 μl of overnight culture in a thermocycler for 10 min at 94°C; cell debris was then briefly pelleted at 4,000 × *g* for 5 min. These extractions were used for screening the large culture collections.

Genome sequencing and assembly. Genomic DNA was sequenced at the Genome Resource Center at the Institute for Genome Sciences (http://www.igs.umaryland.edu/resources/grc/). As part of the GSCID project, 454 paired-end reads (3-kb insertion size) were assembled with the Celera assembler (22). Paired-end Illumina reads from a GA-II platform were also assembled with a reference-guided approach (AMOScmp [23]); contigs were further processed with ABACAS (24) and IMAGE (25) to generate more-contiguous assemblies. Raw sequence reads were then mapped to the reference-guided assembly with bwa (26). To identify potential genome sequences not present in the reference genome, raw reads that failed to map to the reference-guided assembly were quality trimmed with sickle and assembled with Velvet (27). Contigs from the two methods (reference guided, *de novo*) were concatenated, and sequencing errors were corrected with iCORN (28).

Whole-genome alignment and phylogeny. To identify the conserved genomic core, conserved regions in isolates from *E. coli* and *Shigella* species were identified from a Mugsy (29) alignment of a diverse set of reference genomes ($n = 40$) (30). These conserved genomic regions were then extracted from 336 *E. coli* and *Shigella* genomes (see Table S1 in the supplemental material) with BLASTN (31), aligned with MUSCLE (32), and concatenated. A tree was inferred on the basis of reduced alignment with FastTree2 (33), with the following settings: -spr 4 -mlacc 2 -slownni.

A total of 69 *Shigella* genomes were aligned with Mugsy and processed as reported previously (17); this set included the 55 genomes sequenced in this study as well as 14 reference genomes. From the whole-genome alignment, subtractive methods were used to identify blocks of sequence from the output that are unique to monophyletic *Shigella* lineages. This was accomplished by identifying blocks of sequence that were conserved only in the targeted *Shigella* lineage and were absent from all other *Shigella* genomes.

Multigene phylogenies. For a comparison to the whole-genome phylogeny, gene-based trees were also inferred from a concatenation of multilocus sequence typing (MLST) markers (34) (see Table S2 in the supplemental material) informatically extracted from 336 *E. coli* and *Shigella* genomes; a tree was also inferred with FastTree2 from 7 markers described in a previous study of *Shigella* evolution (15). Recombination of aligned markers was tested with Phi (35).

Multiplex PCR screening. PCR primers for the multiplex assay (see Table S3 in the supplemental material) were designed in Primer3 (36) based on *Shigella* phylogenomic markers identified with Mugsy. All PCRs were performed with GoTaq master mix (Promega). For the multiplex PCR, primers were combined as a single mixture with a final concentration of 0.14 μM for each primer set; the primer set for clade S1 was added for a final concentration of 0.28 μM. The touchdown PCR program consisted of an initial denaturation at 94°C for 5 min, followed by 2 cycles of 94°C for 45 s, 68°C for 45 s, and 72°C for 1 min; this was followed by 2 cycles with an annealing temperature of 64°C and then 28 cycles with an annealing temperature of 60°C, keeping all other parameters constant. To determine the specificity of each *Shigella* phylogenomic marker, 6 strain collections were screened with the multiplex assay: the collection of isolates sequenced in this study, a collection of isolates ($n = 42$) from western Kenya (see description above), a collection from the Public Health Agency of Canada ($n = 39$), a collection from Chile ($n = 106$), the environmental *E. coli* collection ($n = 72$) (ECOR) (37), and the diarrheagenic *E. coli* (DECA) collection ($n = 79$) (http://www.shigatox.net/stec/cgi-bin/deca) (38); the isolates from the ECOR collection were characterized by multilocus enzyme electrophoresis, and the DECA isolates have all been sequenced and deposited in public databases.

BSR analysis. A comparison of genes between the *Shigella* and *E. coli* genomes was performed with a BLAST score ratio (BSR) analysis (39, 40). Coding region sequences (CDSs) were predicted independently with Prodigal (41) for 69 *E. coli* and 69 *Shigella* genomes (see Table S1 in the supplemental material); the *E. coli* genomes were randomly subsampled from all available *E. coli* genomes with a Python script (https://gist.github.com/jasonsahl/115d22bfa35ac932d452). All CDSs were translated with BioPython (42) and then clustered with USEARCH v6 (43) at an identity value (ID) of 0.9 to dereplicate the data set. Each unique cluster was then translated with BioPython, and peptides were aligned against their nucleotide sequences with TBLASTN in order to obtain the maximum alignment bit score. The alignment bit score for each gene was divided by the maximum bit score for all genomes in order to obtain the BSR. For the pan-genome calculation, peptides from all genomes were clustered with USEARCH (43) over a range of IDs (0.1 to 1.0). The number of clusters at each identity threshold was calculated and plotted. This procedure was applied to all *Shigella* genomes ($n = 69$) and a subset of randomly selected *E. coli* genomes ($n = 69$) (see Table S1).

O antigen typing of each newly sequenced *Shigella* genome. The nucleotide sequences for all annotated *Shigella* O antigens (9) were downloaded. Each genome was assigned a bioinformatically derived O antigen type based on the BLAST hit most similar to previously characterized O antigen sequences.

Nucleotide sequence accession numbers. Nucleotide sequence data determined in this work have been deposited in GenBank (see Table 1 for accession numbers).

TABLE 1 Strains examined in this study

Isolate name	Species[a]	Clade[b]	MLST type[c]	Predicted O antigen[c]	Data set[d]	No. of contigs	Total no. of bp	GenBank accession no.
SB_4444-74	*S. boydii*	S1	145	O53	GSCID	314	4,976,495	AKNB00000000
SB_08_0009	*S. boydii*	S1	145	*S. boydii* type 2	Canada	165	4,864,228	AMJZ00000000
SB_08_2671	*S. boydii*	S1	145	O53	Canada	185	4,817,878	AMKB00000000
SB_S6614	*S. boydii*	S1	145	O150	Kenya	479	4,610,666	AMJU00000000
SB_S7334	*S. boydii*	S1	145	*S. boydii* type 2	Kenya	249	4,711,626	AMJX00000000
248-1B	*S. boydii*	S1	145	*S. boydii* O	Chile	166	4,788,006	AMKG00000000
SB_08_0280	*S. boydii*	S1	243	*S. dysenteriae* type 9	Canada	124	4,835,559	AMKA00000000
SB_08_6341	*S. boydii*	S1	243	O164	Canada	138	4,800,746	AMKD00000000
SB_09_0344	*S. boydii*	S1	243	*S. boydii* type 2	Canada	174	4,821,210	AMKE00000000
SB_08_2675	*S. boydii*	S1	243	*S. boydii* type 2	Canada	335	4,832,830	AMKC00000000
SB_3594-74	*S. boydii*	S1	1,130	*S. dysenteriae* O	GSCID	96	4,634,068	AFGC00000000
SB_965-58	*S. boydii*	S3	250	*S. boydii* type 15	GSCID	96	5,184,598	AKNA00000000
SB_5216-82	*S. boydii*	S3	1,748	O40	GSCID	75	4,882,454	AFGE00000000
SD_1617	*S. dysenteriae* type 1	S4	146	*S. dysenteriae* O	GSCID	67	4,613,558	ADUT00000000
SD_225-75	*S. dysenteriae* type 2	S1	148	*S. dysenteriae* type 3	GSCID	111	4,813,171	AKNG00000000
SD_S6554	*S. dysenteriae* type 2	S1	243	O150	Kenya	555	4,260,325	AMJS00000000
SD_S6205	*S. dysenteriae* type 2	S3	147	*S. dysenteriae* type 2	Kenya	582	5,069,695	AMJQ00000000
SD_155-74	*S. dysenteriae* type 2	S3	288	*S. dysenteriae* O	GSCID	114	5,162,699	AFFZ00000000
SF_CCH060	*S. flexneri*	S1	145	O13	GSCID	82	4,771,928	AKMW00000000
SF_1485-80	*S. flexneri*	S1	145	F6	GSCID	82	4,680,138	SRX024343
SF_K-315	*S. flexneri*	S1	1,512	O13	GSCID	79	4,564,844	AKMY00000000
SF_2457T	*S. flexneri*	S5	245	O13	GSCID	94	4,807,953	ADUV00000000
SF_K-218	*S. flexneri*	S5	245	O13	GSCID	74	4,885,634	AFGV00000000
SF_K-304	*S. flexneri*	S5	245	O13	GSCID	104	4,698,223	AFGZ00000000
SF_K-404	*S. flexneri*	S5	245	O13	GSCID	91	4,836,578	AKMZ00000000
SF_K-671	*S. flexneri*	S5	245	O13	GSCID	82	4,702,647	AFHA00000000
SF_2747-71	*S. flexneri*	S5	245	O13	GSCID	50	4,656,186	AFHB00000000
SF_2930-71	*S. flexneri*	S5	245	O13	GSCID	50	4,644,642	AFHD00000000
SF_4343-70	*S. flexneri*	S5	245	O13	GSCID	63	4,320,710	AFHC00000000
SF_S5644	*S. flexneri*	S5	245	O13	Kenya	491	4,689,099	AMWM00000000
SF_S5717	*S. flexneri*	S5	245	O13	Kenya	222	4,805,235	AMJP00000000
SF_S6585	*S. flexneri*	S5	245	O13	Kenya	274	4,815,370	AMJT00000000
SF_S6678	*S. flexneri*	S5	245	O13	Kenya	247	4,798,162	AMJV00000000
SF_S6764	*S. flexneri*	S5	245	O13	Kenya	402	4,673,236	AMJW00000000
SF_S7737	*S. flexneri*	S5	245	O13	Kenya	288	4,583,415	AMJY00000000
SF_K-227	*S. flexneri*	S5	628	O13	GSCID	77	4,804,544	AFGY00000000
SF_K-272	*S. flexneri*	S5	628	O13	GSCID	71	4,510,649	AFGX00000000
SF_2850-71	*S. flexneri*	S5	628	O13	GSCID	53	4,787,407	AKMV00000000
SF_J1713/17B	*S. flexneri*	S5	629	O13	GSCID	52	4,729,738	AFOW00000000
SF_S6162	*S. flexneri*	S5	630	O13	Kenya	869	4,669,004	ANAN00000000
SF_6603-63	*S. flexneri*	S5	1,022	O13	GSCID	66	4,626,909	SRX023788
SF_VA-6	*S. flexneri*	S5	1,025	O13	GSCID	70	4,679,117	AFGW00000000
SF_K-1770	*S. flexneri*	S5	1,025	O13	GSCID	101	4,814,276	AKMX00000000
MT1457	*S. flexneri*	S5	1,753	O58	Chile	341	4,552,076	AMKF00000000
SS_Moseley	*S. sonnei*	S2	152	O58	GSCID	115	5,145,982	SRX024338
SS_53G	*S. sonnei*	S2	152	O58	GSCID	176	5,188,167	ADUU00000000
SS_3226-85	*S. sonnei*	S2	152	O58	GSCID	108	4,976,082	AKNC00000000
SS_3233-85	*S. sonnei*	S2	152	O58	GSCID	72	4,997,922	AKND00000000
SS_4822-66	*S. sonnei*	S2	152	O13	GSCID	91	4,710,354	AKNE00000000
SS_08_7765	*S. sonnei*	S2	152	O58	Canada	55	4,885,496	AMKI00000000
SS_08_7761	*S. sonnei*	S2	152	O58	Canada	95	4,893,159	AMKH00000000
SS_09_1032	*S. sonnei*	S2	152	O58	Canada	110	4,924,296	AMKJ00000000
SS_09_4962	*S. sonnei*	S2	152	O58	Canada	86	4,883,415	AMKL00000000
SS_09_2245	*S. sonnei*	S2	152	O58	Canada	92	4,862,336	AMKK00000000
SS_S6513	*S. sonnei*	S2	152	O58	Kenya	234	4,843,790	AMJR00000000

[a] Data were determined by serology.
[b] Clade names are from the current study.
[c] Data were determined informatically.
[d] GSCID, Genome Sequencing Center for Infectious Diseases.

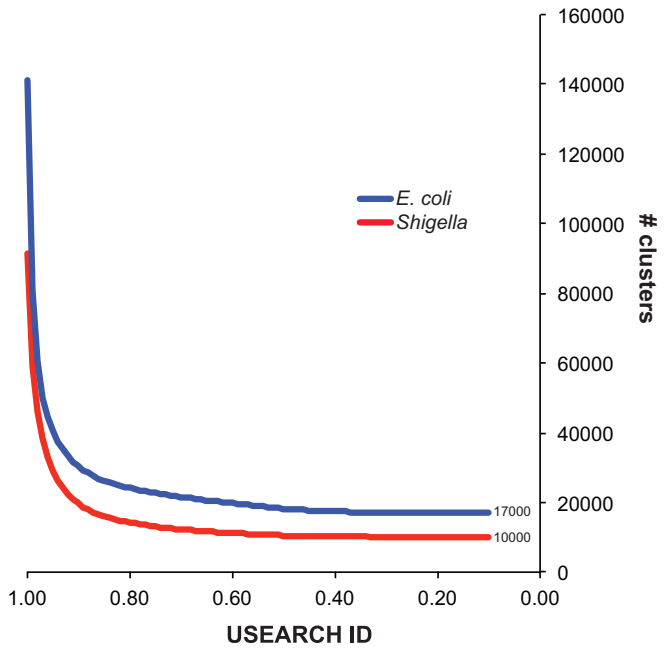


FIG 1 A plot of unique gene clusters at different levels of identity. Coding regions for 69 *Shigella* genomes or 69 *E. coli* genomes were predicted with Prodigal (41). Coding regions were translated with BioPython (42) and concatenated. USEARCH (43) was then used to cluster all peptides at different levels of identity. The number of unique clusters at each identity threshold was plotted for both groups. The results demonstrate the smaller pan-genome size for *Shigella* compared to *E. coli* genomes.

RESULTS

Pan-genome comparisons between *Shigella* and *E. coli*. The predicted size of the *Shigella* pan-genome, based on an analysis of 69 draft and finished genomes, is ~10,000 genes (USEARCH ID threshold of ~0.40% to ~40% protein identity over 100% of the peptide) (Fig. 1); this number is relatively small, considering that each *Shigella* genome contains ~5,050 predicted coding regions as determined on the basis of default settings in Prodigal (41). The pan-genome for *E. coli*, based on the same threshold, is significantly larger (~17,000 genes) (44). This difference may be indicative of the large number of environments where *E. coli* can be isolated, in contrast to *Shigella* species, which are primarily identified as pathogens of humans.

The numbers and compositions of genes were also compared using BLAST score ratio (BSR) analysis (40, 45). The core genome of *E. coli*, based on an analysis of 69 genomes, is ~2,155 genes (BSR ≥ 0.80 in 100% of the genomes). This number is consistent with a previous calculation (44, 46) based on a smaller number of genomes. The core conserved *Shigella* genome consists of ~1,880 genes (BSR ≥ 0.80 in 100% of the genomes); a list of accession numbers for the genes that are conserved in the *E. coli* and *Shigella* pan-genome is provided in Table S4 in the supplemental material. A comparison of genes between both pan-genomes demonstrates that only 1,447 genes are shared by all *E. coli* and *Shigella* isolates. This finding provides the insight that *Shigella* species do not have the same genomic profile as *E. coli*.

Genomic comparison of *E. coli* and *Shigella* genomes. To identify genes differentially distributed between the *E. coli* and *Shigella* genomes, a large-scale BSR (LS-BSR) analysis was performed on 69 *E. coli* and 69 *Shigella* genomes (45). The results demonstrate that several genes, primarily associated with metabolism, are conserved in *E. coli* isolates and largely absent ($n < 2$) in *Shigella* isolates (Table 2); this stands in contrast to a recent study which suggested that no genes could be used to distinguish the two groups (47). In fact, some of the genes identified as being differentially present in *E. coli* and not in *Shigella* have been previously identified as being pathoadaptive for *Shigella* (48), suggesting that the analysis is valid. Genes were also identified that are differentially conserved in *Shigella* genomes; these include those encoding a siderophore receptor, an invasion plasmid antigen, and several hypothetical proteins (Table 2). These genes appear to be involved in pathogenesis (49), suggesting a niche specialization of *Shigella* compared to *E. coli*. The BSR matrix for these comparisons is publically available (https://github.com/jasonsahl/shigella_BSR_matrix).

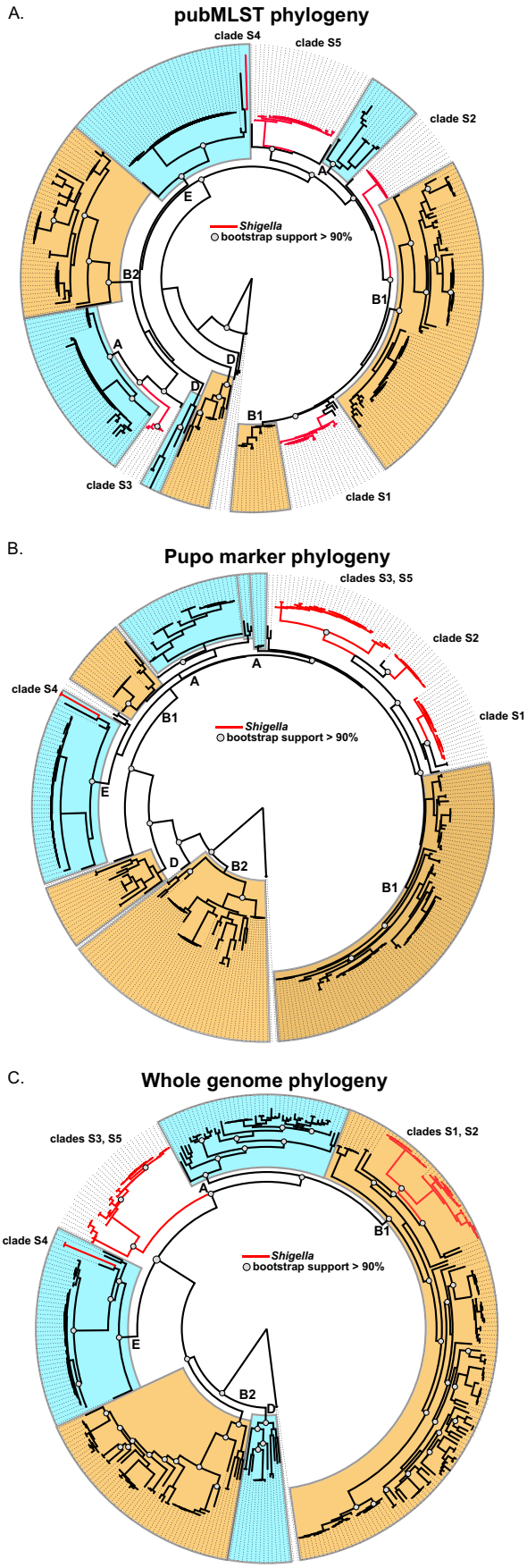
Multigene phylogenies. Previous conclusions regarding *Shigella* evolution were based on a concatenation of a small set of conserved genomic loci. To evaluate the topology of trees inferred from concatenated multigene alignments, a phylogeny was inferred from ~3.5 kb of concatenated MLST sequences using the pubMLST system (34). The resulting MLST-based phylogeny indicates that *Shigella* has emerged from *E. coli* on five separate occasions (Fig. 2A). A phylogeny inferred from seven concatenated markers (~7 kb) used in a previous study of *Shigella* evolution (15) indicates that the *Shigella* genotype has emerged from *E. coli* on a minimum of four separate occasions (Fig. 2B). These findings highlight the variability in phylogenetic placement based on the composition of the input sequence data.

Whole-genome-alignment phylogeny. A whole-genome-based phylogeny was inferred for all *E. coli* and *Shigella* genomes, including the 55 new *Shigella* genomes sequenced in this study and 14 *Shigella* assemblies in GenBank (Fig. 2C). The resulting phy-

TABLE 2 Differences in BSR values in features between the *E. coli* and *Shigella* genomes

Locus tag	Avg BSR[a]		Annotation
	E. coli (n = 69)	*Shigella* (n = 69)	
EcE24377A_0358	**0.98**	0.04	2-Methylcitrate dehydratase
ECO5905_06979	**0.98**	0.06	Cytosine permease
EcHS_A0402	**0.98**	0.06	Cytosine deaminase
HMPREF9530_02672	**0.97**	0.14	Methylisocitrate lyase
ECNG_01839	**0.86**	0.08	Hypothetical protein
ECSTEC94C_0398	**0.9**	0.11	Lactose permease
ECAA86_00424	**0.96**	0.23	2-Methylcitrate synthase
ECSE_0359	**0.99**	0.26	Propionyl-CoA synthetase
UTI89_C0362	**0.98**	0.28	Hypothetical protein
EIQ72748	**0.97**	0.24	Protein PrpR
EcSMS35_0562	**0.99**	0.22	Ureidoglycolate dehydrogenase
SBO_4341	0.24	**0.97**	Ferric siderophore receptor
SDY_P140	0.11	**0.83**	Invasion plasmid antigen
SbBS512_E0714	0.41	**0.99**	Hypothetical protein
SFK671_1049	0.29	**0.89**	Hypothetical protein
Sd1012_0960	0.26	**0.93**	Hypothetical protein

[a] Boldface indicates values that are ≥0.8 in one group and <0.4 in the other, indicating genes that are highly conserved in one group and absent or significantly divergent in the other.



logeny illustrates the phylogenetic placement of *Shigella* genomes in the context of a diverse set of *E. coli* genomes. Clades S1 and S2 form a monophyletic clade, as do clades S3 and S5. Clade S4, which includes only *S. dysenteriae* type 1 isolates, is closely related to O157:H7 enterohemorrhagic *E. coli* (EHEC) isolates, as has been demonstrated previously (46, 50).

The conserved genomic core, based on a whole-genome alignment of 69 *Shigella* genomes, consists of ~2.4 Mb of homologous sequence data. A phylogeny based on this whole-genome alignment demonstrates the presence of five clearly defined monophyletic *Shigella* clades (S1 to S5) (Fig. 3). The *S. flexneri*, *S. boydii*, and *S. dysenteriae* isolates, as defined by serology studies, did not follow a monophyletic genomic distribution within these five clades. Although *Shigella* isolates grouped into three clades in the comparative studies with *E. coli*, the *Shigella*-only comparisons provide subclade designations that allow improved discrimination of *Shigella* genomes based on genomic content.

Identification of *Shigella* clade-specific genomic regions. When the 69 *Shigella* genomes were aligned using Mugsy (29), no universally conserved genomic regions could be identified for any *S. flexneri*, *S. boydii*, or *S. dysenteriae* isolates. Therefore, an approach was employed to consider gene conservation in each of the five phylogenomic clades in the *Shigella*-only whole-genome phylogeny (Fig. 3), regardless of species designations based on previous identification by serotyping. Genomic regions were identified from the Mugsy alignment that were unique to each of the five clades.

The phylogenetic reconstruction clearly demonstrates that clades S1 and S3 contain a mixture of *Shigella* species, as defined by traditional typing, including serological methods (Fig. 3). To confirm that these anomalous genomes had not been mistyped, the closest O antigen for each genome was determined informatically (9) (Table 1). The results demonstrate that the bioinformatics-based serotyping is congruent with the laboratory-determined serotype. For example, there are four *S. flexneri* genomes that are included in clade S1 (Fig. 2A). A BLAST search demonstrated that each of these genomes contains the *S. flexneri* 6 O antigen (9), while all *S. flexneri* isolates from clade S5 contain the O13 antigen. The phylogeny demonstrates that genomes with the *S. flexneri* 6 O antigen are not closely related to sequenced *S. flexneri* genomes with the O13 antigen as determined on the basis of genomic content. This example highlights the difference between the phenotypic markers and the genotypic markers, which we can now integrate into the identification algorithm.

To identify the conservation of phylogenomic markers, a set of 96 *S. flexneri* genomes from a separate study (see Table S1 in the

FIG 2 Phylogenies inferred from a diverse set of *E. coli* and *Shigella* genomes ($n = 336$). (A) A phylogeny inferred from a concatenation of sequences from multilocus sequence typing markers (see Table S3 in the supplemental material) from the *E. coli* pubMLST system (34). Conserved sequences were extracted from BLAST (31) alignments and were aligned with MUSCLE (32). The phylogenetic tree was inferred with FastTree2 (33), with 1,000 bootstrap replicates. (B) A phylogeny was inferred with FastTree2 from a concatenation of sequence markers (see Table S2) used in a previous study of *Shigella* evolution (15). (C) A phylogenetic tree of *E. coli* and *Shigella* isolates using whole-genome sequence data. Conserved genomic fragments were first identified in a core set of 40 *E. coli* genomes aligned with Mugsy (29). Conserved genomic regions were extracted by BLASTN, aligned with MUSCLE, and concatenated. A tree was then inferred on this alignment with FastTree2, with 1,000 bootstrap replicates.

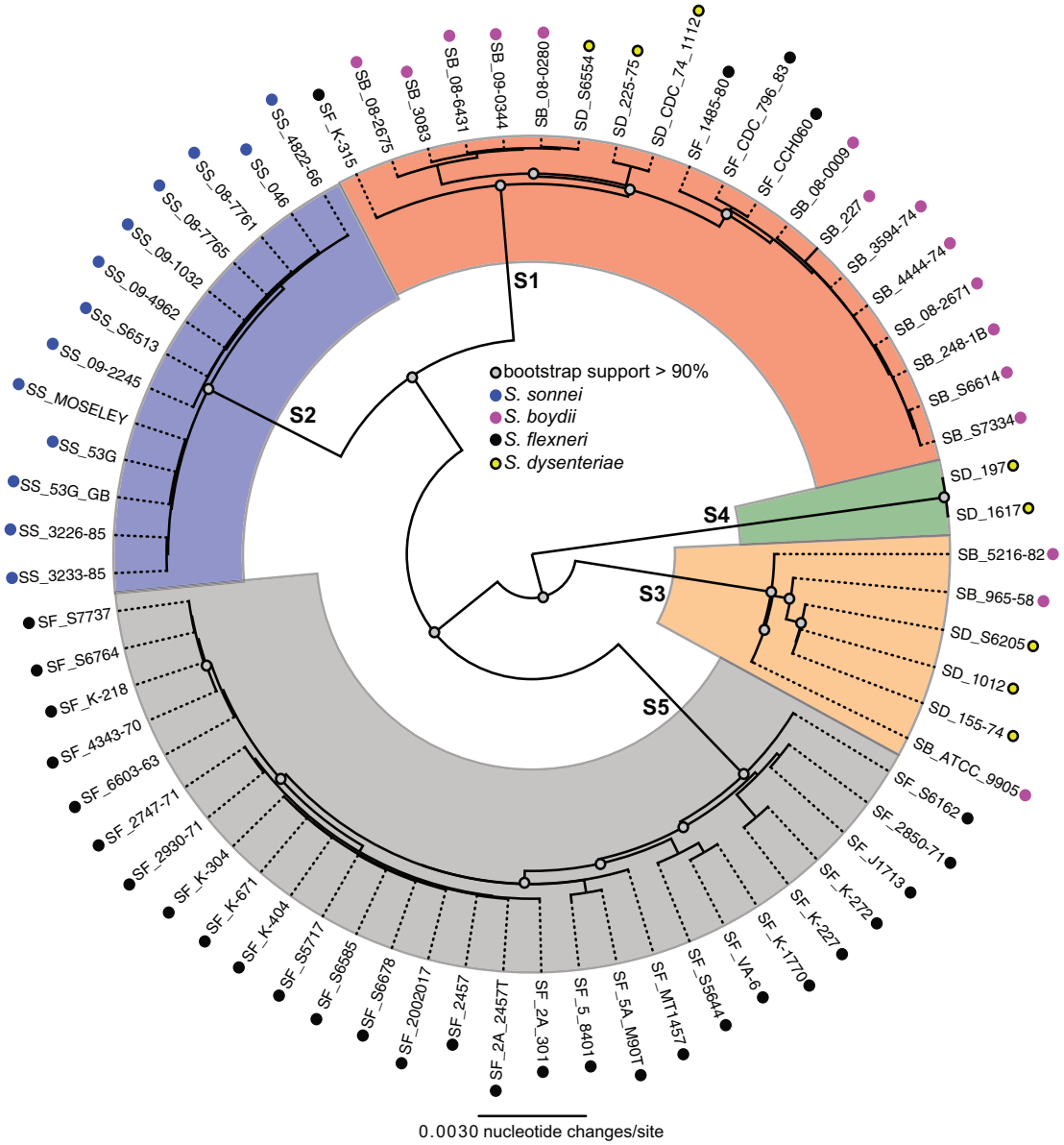


FIG 3 Whole-genome phylogeny. A whole-genome phylogenetic tree of 69 sequenced *Shigella* genomes, including 55 sequenced as part of this study, is shown. The tree was inferred with FastTree2 (33) on a Mugsy (29) whole-genome alignment, as has been done previously (17). Labels at branch nodes indicate the clade-naming convention developed in this study. Bootstrap support values from 100 replicates are shown at nodes. This tree demonstrates that *Shigella* genomes group into 5 monophyletic lineages (S1 to S5) and that there is a mixing of species, based on serology, in clades S1 and S3.

supplemental material) were queried with conserved *S. flexneri* regions identified in this study. All 96 genomes contained the S5 marker, a finding which supports the idea of the specificity of this marker for *S. flexneri* genomes in this genomic context (Fig. S1 in the supplemental material).

PCR assay development. PCR assays were developed to amplify conserved genomic regions from each clade in the *Shigella* phylogeny (Fig. 3; see also primer sequences in Table S3 in the supplemental material). A PCR assay of the 55 isolates sequenced in this study demonstrated that a single amplicon was produced for each isolate as visualized by gel electrophoresis; the size of the band corresponded to the conserved genomic fragment designed for each clade (Fig. 3 and 4). To investigate the specificity and

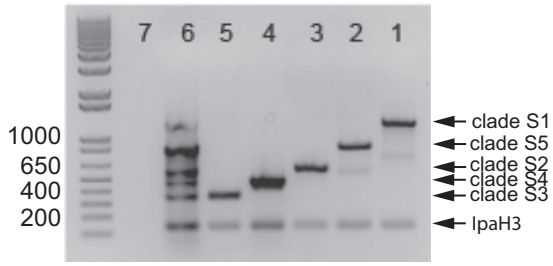


FIG 4 *Shigella* biomarker development. A gel electrophoresis image of amplicons from the 5 major clades identified in this study (lanes 1 to 5) is shown; two bands, one genus targeted (*ipaH3*) and one clade targeted (S1 to S5), indicate a positive reaction. Lane 6 shows a coinfection reaction with all 5 clades, plus the universally conserved *ipaH3* marker. Numbers on the left represent numbers of base pairs in the DNA ladder.

TABLE 3 Results of the multiplex PCR assay

Isolate collection or parameter[a]	No. of isolates			
	Total	0 bands	1 band	2 bands
Kenyan	42	0	0	42
Canadian	39	0	0	39
Chilean	106	1	2	103
MSU/STEC Center	34	0	0	34
ECOR	72	70	2	0
DECA	79	75	4	0
Total no. of isolates screened	372			

[a] MSU, Michigan State University; STEC, Shiga-toxigenic *Escherichia coli*; ECOR, environmental *E. coli* collection; DECA, diarrheagenic *E. coli* collection.

sensitivity of the *Shigella* PCR assay, additional culture collections were examined, and the results demonstrated that the conserved markers were present in temporally and geographically diverse *Shigella* isolates (Table 3). However, a PCR screen of two *E. coli* isolate collections demonstrated positive amplification of some target regions in a small number of *E. coli* isolates (Table 3). Therefore, the assay was redesigned to increase the specificity for *Shigella* genomes by adding a second, species-specific amplicon.

From the Mugsy alignment, genomic fragments were identified that were conserved in all 69 sequenced *Shigella* genomes and absent in *E. coli*. A BLAST search of these putative markers against a curated database of *E. coli* genomes identified a number of conserved *Shigella* markers in all 69 *Shigella* sequences and absent in the curated *E. coli* collection. One genomic fragment (240 bp) of the invasion antigen IpaH3 was found to be conserved in all *Shigella* genomes and also enteroinvasive *E. coli* (EIEC) isolates 53638 (NZ_AAKB00000000) and LT-68 (ADUP00000000). However, these EIEC isolates do not contain any of the *Shigella* clade-specific markers.

PCR primers designed from the IpaH3 marker were added to a mixture containing primers for all five clades. In this new multiplex assay, two bands, one for the IpaH3 marker and an additional band for the phylogenomic clade-specific marker, are required for identification of the isolate as positive for *Shigella* (Fig. 4). The assay can also potentially identify coinfections, where isolates from multiple clades are potentially present in a single sample (Fig. 4, lane 6). Six strain collections, totaling 372 isolates, were PCR screened with this multiplex PCR assay. The results demonstrated that, of 221 *Shigella* isolates collected from diverse geographic locations, 218 produced two distinct bands, indicating identification of both "genus" and phylogenomic clade. Furthermore, of 151 *E. coli* isolates examined, none produced two amplicons (Table 3). Three putative *Shigella* isolates failed to produce 2 bands, which gives a false-negative rate of 1.4% and a sensitivity of 98.6%; two of these negative isolates were serotyped as *S. boydii* and one isolate was typed as *S. dysenteriae*. Phylogenetic analysis and further genome sequencing are required to confirm the identity of these isolates.

Subclade typing. In addition to the five major *Shigella* clades, PCR assays were also designed to identify *Shigella* genomes that did not group with other representative genomes of the same species (see Table S3 in the supplemental material). An additional PCR screen of 105 *Shigella* isolates from the Chilean collection identified three isolates that were typed as *S. flexneri* but belong to clade S1, which contains *S. boydii*, *S. dysenteriae*, and *S. flerxneri*, based on the multiplex assay. A PCR assay using primers designed for the *S. flexneri* 6 O antigen biosynthetic cluster demonstrated positive amplification for each of the three *S. flexneri* clade S1 isolates. PCR assays were also designed for *S. dysenteriae* genomes in clade S1, *S. boydii* genomes in clade S3, and *S. dysenteriae* genomes in clade S3. Although these markers are not unique to targeted genomes in each clade, they may be used for differentiation of the species, as defined by traditional serotyping, within a given phylogenomic clade.

DISCUSSION

Shigella species are intracellular human pathogens that can cause serious, potentially lethal intestinal disease, primarily in the developing world, with wide-ranging clinical manifestations, including tenesmus, abdominal pain, and bloody, mucous-like, or watery diarrhea (1) (4). On the basis of sequencing of a small number of genomic loci, *Shigella* species have been thought to have emerged from *Escherichia coli* on at least seven separate occasions. However, by analysis of the core, conserved genome, a higher-resolution analysis of evolution can be performed. The results of a whole-genome alignment and phylogenetic method utilizing 336 *E. coli* and *Shigella* genomes (Fig. 2C) clearly demonstrate that all *Shigella* isolates sequenced to date group into 3 monophyletic clades; this demonstrates that *Shigella* clades S1 and S2 are more similar to each other than they are to those of other *E. coli* isolates. Figure 2 also demonstrates the close relatedness of *Shigella* genomes, especially within clades S3 and S5.

A study by Pupo et al. divided *Shigella* isolates into 3 monophyletic clades, with 5 outliers, based on a phylogenetic analysis of ~7 kb of concatenated sequence (15). Those authors concluded that the *Shigella* phenotype has arisen seven times, not counting the divergent *S. boydii* 13 isolate (15). Recent evidence has demonstrated that *S. boydii* 13 is not invasive and is therefore likely not similar or related to other *Shigella* species (51). In the current study, markers used in the study by Pupo et al. were informatically extracted from genome assemblies and used to infer a phylogeny from 336 *E. coli* and *Shigella* genomes (Fig. 2B). Four *E. coli* genomes were identified in this phylogeny that grouped with *Shigella* clades and that did not group with *Shigella* clades in the whole-genome phylogeny (Fig. 2B and C). Using the Phi test for recombination (35), the current study demonstrated that at least two of the markers (*thrC* and *trpC*) show signs of recombination (P value < 0.001), which may explain this incongruent topology. This highlights the difficulty with using small amounts of genetic material for the inference of genomic relatedness.

In one other study, a phylogeny was inferred from a concatenation of 345 coding regions in 25 genomes that did not show evidence of recombination; the phylogeny revealed that the *Shigella* genomes fell into 2 defined lineages (52). Additionally, a recent study used k-mer frequency clustering of 36 finished genomes to infer a phylogeny and showed that *Shigella* genomes grouped into two monophyletic clades (53). Although our results suggest the presence of three clades, all methods suggest a less diverse evolutionary history for *Shigella* in the broader context of a significant number of *E. coli* isolates. The MLST phylogeny did a relatively poor job of recapitulating the whole-genome *Shigella* phylogeny (Fig. 2A), as has been demonstrated previously (17).

Typically, *Shigella* identification is based on serological or biochemical measures in the field or laboratory (1). Based on the species concept utilized by ecologists, a species of *Shigella* would

be expected to follow a monophyletic history (54). However, the results of our phylogenetic reconstruction based on genomic content demonstrate that *Shigella* species, based on serotype analysis, are not restricted to a particular phylogenomic clade. A previous study also demonstrated mixing of *Shigella* species across phylogenetic clades (15). This finding may have been due to a lack of consensus in strains chosen for antiserum grouping (10) and demonstrates the need for a more comprehensive genomic-based assay to understand the phylogenetic history of *Shigella*.

In addition to the examination of the evolutionary history, whole-genome sequencing and comparative genomic analyses have provided the opportunity to develop a robust PCR-based typing assay. In the present study, a single multiplex PCR assay, designed to produce one genus-targeted and one phylogenomic-clade-targeted amplicon, was developed based on a large-scale comparative genomics analysis. Based on the PCR screening of 218 *Shigella* isolates, the assay appears to universally and specifically amplify *Shigella*. This assay will be a valuable tool to examine both new clinical isolates and existing *Shigella* culture collections. One limitation to this assay is that new and emergent *Shigella* isolates may lack one or more of these genetic markers; however, this is the same limitation that would exist for serotyping isolates with previously uncharacterized O antigens. Additional genome sequencing will improve the understanding of the conservation and distribution of genetic markers, which will help in the continued design and verification of PCR primers for diverse and emergent isolates.

PCR assays have been used previously to detect *Shigella* in a variety of media (55). A recent study proposed a multiplex PCR assay to differentiate only *S. sonnei* and *S. flexneri* isolates (56) but did not factor in the remaining species. The assay presented in our study improves on this proposed multiplex assay by generating a single amplicon per genomic clade and targeting specific genomic sequences that are conserved in all *Shigella* species. The IpaH invasion antigen targets used in this study have previously been used to amplify and quantify *Shigella* isolates (57, 58). The primer set developed in this study was utilized because it amplifies a larger product than the previously published primer pair.

Shigella isolates contain a genome significantly smaller than most related *E. coli* genomes (50); the loss of genes is characteristic of an intracellular pathogenic lifestyle (59). In *Shigella*, genes that potentially interfere with pathogenesis are prone to deletion (60). Many deleted genes have been associated with cellular metabolism (15); these observations were verified by a comparative genomics analysis conducted in the current study (Table 2).

Whole-genome sequence data are an invaluable tool for the study of bacterial pathogens. In this study, genome sequence data were used to refine the evolutionary history of *Shigella* and focus the design of a multiplex PCR assay to characterize isolates. This method represents a new paradigm in which genome sequence data are utilized to better characterize and monitor important human pathogens.

ACKNOWLEDGMENTS

We thank Miles Majcher and Helen Tabor for assistance in provision of strains as well as the Canadian provincial laboratories that provided strains: British Columbia Centre for Disease Control Public Health Microbiology & Reference Laboratory, Alberta ProvLab, Cadham Provincial Laboratory (Manitoba), Ontario Public Health laboratories, and the hospital laboratories of New Brunswick.

This project was funded in part by federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract number HHSN272200900009C and NIH grant number 1U19AI090873. C.R.M. was a trainee under Institutional Training Grant T32AI007540 from the National Institute of Allergy and Infectious Diseases. Additionally, J.W.S., C.R.M., and D.A.R. are supported by funds from the state of Maryland.

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