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Evaluation of two multi-locus sequence typing schemes for commensal *Escherichia coli* from dairy cattle in Washington State

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Abstract

Multi-locus sequence typing (MLST) is a useful system for phylogenetic and epidemiological studies of multidrug-resistant *E. coli*. Most studies utilize a seven-locus MLST, but an alternate two-locus typing method (*fumC* and *fimH*; CH typing) has been proposed that may offer a similar degree of discrimination at lower cost. Herein, we compare CH typing to the standard seven-locus method for typing commensal *E. coli* isolates from dairy cattle. In addition, we evaluated alternative combinations of eight loci to identify combinations that maximize discrimination and congruence with standard seven-locus MLST among commensal *E. coli* while minimizing cost. We also compared both methods when used for typing uropathogenic *E. coli* (UPEC). CH typing was less discriminatory for commensal *E. coli* than the standard seven-locus method (Simpson's Index of Diversity = 0.933 [0.902-0.964] and 0.97 [0.96-0.979], respectively). Combining *fimH* with housekeeping gene loci improved discriminatory power for commensal *E. coli* from cattle but resulted in poor congruence with MLST. We found that a four-locus typing method including the housekeeping genes *adk*, *purA*, *gyrB* and *recA* could be used to minimize cost without sacrificing discriminatory power or congruence with Achtman seven-locus MLST when typing commensal *E. coli*.

Abbreviations

CH typing, (*fumC*-*fimH*) typing; ESBL, extended-spectrum β -lactamase; MLST, multi-locus sequence typing; UPEC, uropathogenic *E. coli*.

Keywords: MLST; CH typing; *fimH*; housekeeping genes; *E. coli*

1. Introduction

Pathogenic *Escherichia coli* can cause extraintestinal infections, including urinary tract infections, bloodstream infections, and meningitis (Kaper *et al.*, 2004). Community and hospital-acquired ESBL-producing *E. coli* are increasingly frequent; in addition to resistance to β -lactams, these pathogens are also resistant to other classes of antimicrobial drugs (Ben-Ami *et al.*, 2009; Doi *et al.*, 2010; Pitout and Laupland, 2008; Toval *et al.*, 2014). CTX-M enzymes are the most prevalent ESBLs worldwide (Canton and Coque, 2006; Canton *et al.*, 2012). Recently, relatively high prevalences of commensal *E. coli* carrying *bla*_{CTX-M} have been detected in U.S. livestock populations (Davis *et al.*, 2015; Mollenkopf *et al.*, 2012). To understand the role of bacterial dissemination in population-level changes in antimicrobial resistance, and to discover the sources and transmission routes for *bla*_{CTX-M}-carrying commensal *E. coli* strains isolated from different farms, an effective and reliable strain-typing method is needed.

Typing methods that are based on DNA sequence variation of slowly evolving housekeeping genes have been used for epidemiological studies and to determine evolutionary relationships among *E. coli*, including ESBL-producing strains (Cooper and Feil, 2004; Maiden, 2006; Urwin and Maiden, 2003). The Achtman seven-locus scheme is the most frequently used (Clermont *et al.*, 2015; Wirth *et al.*, 2006). Analysis of sequence types and clonal complexes (closely related sequence types) enables identification of a specific clone or clones that are associated with certain *bla*_{CTX-M} genes. For example, *E. coli* ST131 is a clonal group of pathogenic *E. coli* that is associated with *bla*_{CTX-M-15} (Livermore, 2012; Nicolas-Chanoine *et al.*, 2008; Peirano and Pitout, 2010). Depending on the number of loci included in the assay, conventional MLST assays can be expensive and may lack discriminatory power because of the slow accumulation of genetic variation across housekeeping genes (Overdevest *et al.*, 2012).

A two-locus typing scheme was developed for clinical *E. coli* isolates based on a housekeeping gene (*fumC*) and a virulence gene (*fimH*). This scheme displayed a greater discriminatory power compared to seven-locus MLST and was able to clearly differentiate clinical *E. coli* isolates at a lower cost (Weissman *et al.*, 2012). We evaluated the suitability of the CH typing scheme as a tool to discriminate among CTX-M-carrying commensal *E. coli* from cattle, and compared the discriminatory power of different combinations of loci, as well as their correspondence with the Achtman seven-locus scheme, for assessing diversity and clonal relationships among commensal *E. coli* from cattle.

2. Materials and methods

2.1. Bacterial isolates:

We selected 150 *E. coli* isolates that were obtained during a previous study of *E. coli* carrying *bla*_{CTX-M} on dairy farms in Washington State (Davis *et al.*, 2015). *E. coli* isolates were archived at - 80°C in buffered glycerol for long-term storage. The presence of *bla*_{CTX-M} was determined using published PCR protocols (Edelstein *et al.*, 2003; Mollenkopf *et al.*, 2012). For the current study *bla*_{CTX-M}-positive *E. coli* were chosen randomly from one to three pre-weaned calves and adult cows on each of 27 farms (total n=75). Isolates that were *bla*_{CTX-M}-negative were available from 18 of those 27 farms and from each of those we chose one to six isolates randomly from pre-weaned calves and adult cows. Randomization was performed using the RAND function in Microsoft Excel (Redmond, WA, USA). The result was a set of 150 isolates including 75 with *bla*_{CTX-M} and 75 lacking *bla*_{CTX-M} from 27 and 18 farms, respectively.

To determine whether the combination of MLST loci optimized for commensal *E. coli* from cattle would be suitable for clinical *E. coli*, we used 27 multidrug resistant (MDR) *E. coli*

isolates associated with urinary tract infections in dogs hospitalized at the Washington State University Veterinary Teaching Hospital (Pullman, Washington, USA) between October 2009 and October 2013. This analysis also incorporated the MLST and *fimH* sequence data for 23 UPEC strains (6 dog and 17 human strains) from published data (Weissman *et al.*, 2012).

2.2. Nucleic acid extraction:

Single colonies were inoculated into brain heart infusion (BHI) broth (3 ml) and these cultures were incubated overnight at 37°C. Genomic DNA was isolated using Qiagen DNeasy blood and tissue kit according to manufacturer's instructions (Qiagen Sciences Inc., Germantown, MA). All DNA preparations were stored at 4°C.

2.3. MLST

Seven-locus MLST was performed using published criteria and primers (Wirth *et al.*, 2006). Internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) were amplified in individual reactions and amplification products were submitted to a commercial sequencing service (Functional Biosciences, Inc, Madison, WI). The same primers were used for amplification and bi-directional sequencing [see http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html (Wirth *et al.*, 2006)]. The raw trace files were evaluated and assembled into contigs using BioNumerics v. 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). The allelic profile for each isolate was determined using the BioNumerics MLST Plug-in in accordance with the Achtman scheme available at <http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>. Sequence traces of novel alleles were submitted to the database curator for new allele and sequence type (ST) assignment. Isolates were classified as

the same ST if their alleles were identical at all seven loci. For the purposes of this report, the term “MLST” will be used to denote the Achtman seven-locus scheme.

2.4. *fimH* sequencing and CH typing analysis

fimH amplification and sequencing was done as previously published (Weissman *et al.*, 2012) using the primers: *fimH*-F, CACTCAGGGAACCATTCAGGC; *fimH*-R, CTTATTGATAAACAAAAGTCAC. Cycling conditions for all reactions consisted of 1 cycle of 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 15 s, and 72°C for 1 min. Contigs were assembled using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI). Allele assignments for *fimH* were determined by one of the coauthors (SJW). In cases when the PCR results were negative for the expected 975 bp product for *fimH*, the allele was assigned allele number 0. Sequence traces of novel *fimC* alleles were submitted to the MLST database curator for new allele assignment. The combination of *fimC* and *fimH* allele designations were used as the CH “type.”

2.5. Discriminatory power analyses and clustering comparison coefficients

The discriminatory power of each typing method was evaluated using Simpson's index of diversity (*D*-index). The *D*-index is a measure of the probability that two randomly sampled isolates from a population belong to different types. The index ranges between 0 and 1 and a value ≥ 0.95 is interpreted to mean that the method is suitable for molecular typing (van Belkum *et al.*, 2007). We used the online tool at www.comparingpartitions.info to calculate the Simpson's index of diversity (Simpson, 1949).

The Adjusted Rand (*AR*) and Adjusted Wallace (*AW*) coefficients were used to assess the agreement between the different typing methods. The *AR* coefficient is an estimate of the overall concordance between two typing methods after eliminating the degree of concordance that is attributable to chance. The *AW* coefficient measures how well each method predicts the type assignment of the other method, accounting for the amount of agreement expected by chance. We also used the online tool at www.comparingpartitions.info to calculate these two coefficients (Carrico *et al.*, 2006; Severiano *et al.*, 2011). In total we evaluated all possible combinations of eight loci ($2^8 - 1$, or 255 combinations of loci).

3. Results

3.1. Evaluation of the CH typing method for use on commensal *E. coli*.

The 75 *bla*_{CTX-M}-negative *E. coli* isolates, 75 *bla*_{CTX-M}-positive *E. coli* isolates and 50 UPEC strains were partitioned into 49, 26, and 27 MLST profiles (STs) and 48, 24, and 34 CH types (CHTs), respectively.

Table 1: Number of types found, Simpson's index of diversity D values, Adjusted Rand coefficients and Adjusted Wallace coefficients of MLST and CH typing of *bla*_{CTX-M}-negative *E. coli* isolates, *bla*_{CTX-M}-positive *E. coli* isolates and UPEC isolates.

		No. of types	Simpson's index of diversity D^a	Adjusted Rand coefficient ^b	Adjusted Wallace coefficient ^c
<i>bla</i> _{CTX-M} -negative <i>E. coli</i> isolates n=75	MLST	49	0.976 (0.960-0.993)	0.308 (0.146-0.465)	0.274 (0.156-0.392)
	CH typing	48	0.981 (0.970-0.992)		0.350 (0.207-0.493)
<i>bla</i> _{CTX-M} -positive <i>E. coli</i> isolates n=75	MLST	26	0.94 (0.918-0.962)	0.416 (0.269-0.554)	0.849 (0.776-0.922)
	CH typing	24	0.835 (0.757-0.913)		0.275 (0.173-0.378)
UPEC n=50	MLST	27	0.957 (0.933-0.981)	0.664 (0.446-0.905)	0.516 (0.332-0.701)
	CH typing	34	0.976 (0.959-0.992)		0.93 (0.865-0.996)

^a The probability that two strains sampled randomly from a population belong to two different types (95% confidence interval).

^b The overall concordance of two methods taking into account that the agreement between partitions could arise by chance alone (95% confidence interval).

^c $AW_{MLST \rightarrow CH}$ (MLST rows) is the probability that two strains are classified together using CH typing if they have been classified together using MLST; $AW_{CH \rightarrow MLST}$ (CH rows) is the probability that two strains are classified together using MLST if they have been classified together using CH typing (95% confidence interval).

The Simpson's index of diversity (D) for MLST and CH typing were equivalent for *bla*_{CTX-M}-negative *E. coli* isolates and for UPEC strains. In contrast, the discriminatory power of MLST was greater than that of CH typing for *bla*_{CTX-M}-positive *E. coli* isolates. The overall congruence between the two methods, as measured by the AR coefficient, was low for both *bla*_{CTX-M}-negative *E. coli* isolates and *bla*_{CTX-M}-positive *E. coli* isolates but higher for the UPEC isolates. In *bla*_{CTX-M}-negative *E. coli* isolates, Wallace indices indicated that MLST and CH typing were each non-predictive of the type assignment of the other method but for *bla*_{CTX-M}-positive *E. coli* isolates, MLST assignments were more predictive of CH typing assignments than the other way around. This was the reverse for UPEC for which CH typing had higher ability to predict MLST assignments. Thus, CH typing was more discriminatory and concordant with MLST when used to study UPEC, while MLST was equally or more discriminatory than CH typing when used on commensal *E. coli* isolates from cattle. For the commensal *E. coli*, there was a significant difference between the $AW_{MLST \rightarrow CH}$ for *bla*_{CTX-M}-positive and -negative *E. coli*: MLST had a higher ability to predict CH typing assignments for the latter group (Table 1).

3.2. Determining the optimal combination of loci for typing commensal *E. coli*.

We evaluated individual loci as well as all combinations of the eight loci to determine their discriminatory power and agreement with MLST. For both commensal *E. coli* and UPEC, *gyrB* and *fimH* were the two most variable loci and had the highest D values. Also, *gyrB* demonstrated the best congruence with MLST profiles measured by the Adjusted Rand coefficients (Table 2). To evaluate combinations of two to eight loci, we combined the *bla*_{CTX-M}-positive (n=75) and *bla*_{CTX-M}-negative *E. coli* (n=75) isolates into a single group representing commensal *E. coli* isolates (n=150).

152 Table 2: Numbers of types found, *D* values and Adjusted Rand Coefficient to estimate overall congruence between MLST and each
 153 individual locus for cattle commensal *E. coli* isolates and UPEC isolates.

	No. of types found			Simpson's index of diversity <i>D</i> (95% CI)			Adjusted Rand Coefficient (95 % CI)		
	Commensal <i>E. coli</i>		UPEC	Commensal <i>E. coli</i>		UPEC	Commensal <i>E. coli</i>		UPEC
	<i>bla</i> _{CTX-M⁺} (n=75)	<i>bla</i> _{CTX-M⁻} (n=75)	(n=50)	<i>bla</i> _{CTX-M⁺} (n=75)	<i>bla</i> _{CTX-M⁻} (n=75)	(n=50)	<i>bla</i> _{CTX-M⁺} (n=75)	<i>bla</i> _{CTX-M⁻} (n=75)	(n=50)
<i>fimH</i>	18	31	27	0.726 (0.621-0.831)	0.92 (0.879-0.960)	0.952 (0.924-0.980)	0.24 (0.142-0.335)	0.098 (0.011-0.180)	0.476 (0.172-0.781)
<i>gyrB</i>	13	18	19	0.84 (0.801-0.880)	0.85 (0.786-0.913)	0.922 (0.885-0.958)	0.504 (0.327-0.679)	0.242 (0.125-0.365)	0.694 (0.452-0.939)
<i>icd</i>	12	13	12	0.743 (0.666-0.819)	0.841 (0.804-0.878)	0.889 (0.854-0.924)	0.312 (0.215-0.407)	0.242 (0.125-0.365)	0.532 (0.339-0.735)
<i>fumC</i>	12	18	21	0.733 (0.651-0.814)	0.823 (0.758-0.888)	0.949 (0.927-0.970)	0.299 (0.200-0.394)	0.203 (0.057-0.344)	0.91 (0.797-1.000)
<i>mdh</i>	9	10	14	0.637 (0.528-0.747)	0.835 (0.792-0.878)	0.918 (0.896-0.941)	0.202 (0.119-0.282)	0.22 (0.097-0.350)	0.674 (0.449-0.919)
<i>recA</i>	9	11	14	0.667	0.793	0.914	0.227	0.171	0.651

				(0.570-0.764)	(0.744-0.842)	(0.889-0.939)	(0.145-0.306)	(0.063-0.281)	(0.448-0.872)
<i>adk</i>	10	12	18	0.641	0.584	0.932	0.205	0.066	0.767
				(0.575-0.707)	(0.461-0.707)	(0.909-0.955)	(0.136-0.275)	(0.001-0.125)	(0.530-1.000)
<i>purA</i>	12	13	14	0.545	0.578	0.897	0.143	0.065	0.566
				(0.411-0.679)	(0.448-0.708)	(0.855-0.939)	(0.067-0.213)	(0.017-0.112)	(0.338-0.796)

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For typing commensal *E. coli*, maximum discriminatory power did not significantly increase with the addition of >2 loci, and the maximum Adjusted Rand coefficient achievable did not significantly increase with >4 loci (Figures S1-S3). For commensal *E. coli*, none of the combinations of loci that maximized congruence with MLST included the *fimH* gene (Table 3, Figures S1-S3), and the four loci that would maximize both congruence with MLST and discriminatory power were *adk*, *purA*, *gyrB*, and *recA* (Table 3, Figure S1).

3.3. Distribution of CH Types and MLST sequence types among commensal *E. coli* and UPEC.

Commensal *bla*_{CTX-M}-negative *E. coli* isolates were the most diverse group with regard to MLST types, each of which included ≤ 3 CH types, except for ST58 and ST10; and, as would follow, few CH types included more than one MLST type among *bla*_{CTX-M}-negative *E. coli* isolates. Among *bla*_{CTX-M}-positive *E. coli* isolates, ST744 and CH Type 11-54 were strongly associated: of 29 CH Type 11-54 isolates, 12 were ST744 and all ST744 were CH Type 11-54. CH Type 11-54 also included five other MLST types. Among UPEC isolates, ST groups routinely included multiple CH Types, but only CH Type 35-27 included more than one MLST, ST69 and ST597 (Supplemental Table 1).

171 Table 3. Number of types found, *D* values and Adjusted Rand Coefficient with MLST of different combinations of two to eight gene
172 loci chosen to confer maximum congruence with MLST (Adjusted Rand Coefficient) for 150 commensal *E. coli* isolates. Results from
173 50 UPEC isolates are presented for comparison.

Loci	Number of types		Simpson's <i>D</i>		Adjusted Rand Coefficient ^a	
			(95% CI)		(95% CI)	
	Commensal (n=150)	UPEC (n=50)	Commensal (n=150)	UPEC (n=50)	Commensal (n=150)	UPEC (n=50)
<i>gyrB+icd</i>	44	24	0.942 (0.925-0.960)	0.951 (0.928-0.975)	0.676 (0.553-0.797)	0.935 (0.839-1.000)
<i>adk+gyrB+icd</i>	47	27	0.957 (0.946-0.968)	0.957 (0.933- 0.981)	0.818 (0.725-0.914)	1.000 (1.000-1.000)
<i>adk+purA+gyrB+recA</i>	58	27	0.967 (0.957-0.976)	0.957 (0.933-0.981)	0.955 (0.903-1.000)	1.000 (1.000-1.000)
<i>adk+fumC+purA+gyrB+recA</i>	65	27	0.97 (0.960-0.979)	0.957 (0.933-0.981)	1.000 (1.000-1.000)	1.000 (1.000-1.000)
<i>adk+fumC+gyrB+purA+icd+recA</i>	65	27	0.97	0.957	1.000	1.000

			(0.960-0.979)	(0.933-0.981)	(1.000-1.000)	(1.000-1.000)
<i>adk+fumC+icd+mdh+purA+gyrB+recA</i> ^b	65	27	0.97 (0.960-0.979)	0.957 (0.933-0.981)	---	---
<i>fimH+adk+fumC+purA+gyrB+icd+recA+mdh</i>	85	35	0.981 (0.973-0.989)	0.977 (0.961-0.994)	0.759 (0.627-0.898)	0.682 (0.462-0.922)
CH Typing	65	34	0.933 (0.902-0.964)	0.976 (0.959-0.992)	0.36 (0.262-0.454)	0.664 (0.446-0.905)
174	^a Estimate of overall congruence with MLST					
175	^b Achtman seven MLST housekeeping genes					

4. Discussion:

To study the epidemiology of *bla*_{CTX-M}-carrying *E. coli* in Washington State cattle, we needed to genotype as many isolates as possible with maximum validity and genetic resolution, but at minimal cost. A two-locus method utilizing *fimH* and *fumC* sequences (Weissman *et al.*, 2012) had better discriminatory power than MLST and correlated well with MLST when used on a diverse isolate collection; for studies involving hundreds of bacterial isolates, the cost and labor savings with a two-locus method would be significant. Nevertheless, when used on cattle commensal *E. coli*, the discriminatory power of CH typing was lower than when used in UPEC, and the congruence with MLST was poor. We concluded that CH Typing was not an adequate substitute for MLST for typing cattle commensal *E. coli*. Combining *fimH* allele information with subsets of the seven housekeeping gene loci improved discriminatory power, but decreased congruence with MLST (Figures S1-S3). Our analysis of all possible combinations of *fimH*, *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* alleles on commensal *E. coli* from cattle indicated that costs of typing could be minimized without significantly sacrificing discriminatory power by using the four loci *adk*, *purA*, *gyrB* and *recA* (Table 3, Figures S1-S3).

In contrast with commensal *E. coli*, increasing the number of housekeeping gene loci did not add to the discriminatory power or improve congruence with MLST for UPEC isolates (Table 3). The uniformity of *D* values for different numbers of loci in UPEC suggests a close association between *fimH* alleles and MLST type among UPEC, which is consistent with our findings that ST groups routinely encompassed multiple *fimH* alleles, but only one CH Type mapped to more than one ST (35-27 with ST69 and ST597; Supplemental Table). Housekeeping genes are under neutral or stabilizing selection pressures so that genetic variation arises at a relatively low rate (Dan *et al.*, 2014; Jolley and Maiden, 2014), while genes that experience

positive selection pressure, such as *fimH*, evolve more rapidly resulting in greater genetic variation over similar time periods and thus provide greater potential discriminatory power when used to compare strains (Dan *et al.*, 2014; Jolley and Maiden, 2014; Schwartz *et al.*, 2013; Zhang *et al.*, 2014). Point mutations resulting in pathoadaptive amino acid substitutions in *fimH* of UPEC have been identified in multiple studies (Paul *et al.*, 2013; Schwartz *et al.*, 2013; Sokurenko *et al.*, 1998; Weissman *et al.*, 2006). This could explain the finding that UPEC have more *fimH* allele diversity than fecal commensal *E. coli* from cattle, resulting in greater discriminatory ability of CH typing for UPEC strains (Weissman *et al.*, 2012).

The greatest limitation to this study is the potential lack of generalizability to other *E. coli* populations. Our results for commensal isolates may have been biased by the higher proportion of *bla*_{CTX-M}-carrying *E. coli* in our study sample than would be expected from a random sample of commensal cattle fecal *E. coli*. Most if not all studies of *E. coli* molecular epidemiology target a specific sub-population of this ubiquitous and highly diverse species so it would be possible to evaluate our four-locus method for given a sample of isolates from other host sources, geographic location, environmental matrix, and selection criteria. However, our purpose was not to provide a recommendation for MLST typing of all *E. coli*, but to evaluate CH typing for a cattle commensal *E. coli* and to describe a process for maximizing typing value while minimizing cost. Whole-genome sequencing (WGS) is currently becoming more accessible for use on large numbers of bacterial isolates and in the long term may become a standard for epidemiology research as well as outbreak investigation, but the availability of curated, online international databases for MLST (such as the MLST databases at the University of Warwick) currently provide context for broad comparisons that is not yet available for WGS (Clermont *et al.*, 2015; Gilmour *et al.*, 2013).

222 5. Conclusion

223   This study demonstrated that CH typing is less suitable than MLST for typing commensal
224 *E. coli* from cattle. Our findings also suggest that costs of genetic typing of hundreds of field *E.*
225 *coli* isolates could be minimized without sacrificing either discriminatory power or
226 correspondence with MLST by using only four loci, *adh*, *purA*, *gyrB* and *recA*.

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