High-Resolution Melting System to Perform Multilocus Sequence Typing of Campylobacter jejuni

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Abstract

Multi-locus sequence typing (MLST) has emerged as the state-of-the-art method for resolving bacterial population genetics but it is expensive and time consuming. We evaluated the potential of high resolution melting (HRM) to identify known MLST alleles of Campylobacter jejuni at reduced cost and time. Each MLST locus was amplified in two or three sub fragments, which were analyzed by HRM. The approach was investigated using 47 C. jejuni isolates, previously characterized by classical MLST, representing isolates from diverse environmental, animal and clinical sources and including the six most prevalent sequence types (ST) and the most frequent alleles. HRM was then applied to a validation set of 84 additional C. jejuni isolates from chickens; 92% of the alleles were resolved in 35 hours of laboratory time and the cost of reagents per isolate was $20 compared with $100 for sequence-based typing. HRM has the potential to complement sequence-based methods for resolving SNPs and to facilitate a wide range of genotyping studies.

Introduction

Campylobacter jejuni is the leading reported cause of bacterial gastroenteritis in developed countries [1]. The organisms colonize a range of hosts, including domestic animals and wild birds, and fecal shedding readily contaminates ground water [1]. While outbreaks are well documented, most clinical cases represent isolated, sporadic infections for which the source is rarely apparent. Consumption of contaminated food, especially poultry has been considered the most prevalent source [2]; however, recent studies implicate environmental water and unpasteurized milk as potentially important [3].

Multi-locus sequence typing (MLST), a genotyping system based on single-nucleotide polymorphisms (SNPs) of housekeeping genes, has emerged as the state-of-the-art method for resolving bacterial population genetics [4,5]. A recently developed MLST system for C. jejuni [6] indicates that the species is genetically diverse, with a weakly clonal population structure, marked by frequent intra- and interspecies horizontal genetic exchange [6–8]. Some MLST-defined lineages of C. jejuni have been linked to a restricted geographical area [9] or to particular ecological niches, such as bathing beaches [7], water [10], wild birds [11], chickens, pigs, bovines or sheep [12]. Although MLST is highly reproducible, portable, and easy to interpret, it is complex and expensive to perform.

The development of fluorescent DNA binding dyes with improved saturation properties has allowed a more precise assessment of sequence variation based on the analysis of DNA melting curves. This technique, referred to as high resolution melting (HRM), can distinguish single base variation and then has the potential to identify SNPs without the burden of sequencing [13,14]. After PCR amplification, amplicons are subjected to melting curves with a fluorescence monitoring of a saturating dye that does not inhibit PCR. This approach provides a simple, closed-tube, semi-automated and cost-effective method for detecting base substitutions and small insertions or deletions [15]. Merchant-Patel et al. [16] recently reported the application of HRM for typing the flagellin-encoding flaA gene of Campylobacter jejuni; their results demonstrated that the method is both accurate and easy to implement.

In this study, we describe the novel application of an HRM protocol optimized to perform MLST of C. jejuni isolates. Our goal was to resolve the C. jejuni sequence types as defined in the existing MLST database (http://pubmlst.org/campylobacter) at substantially lower cost than the conventional sequence-based method.

Results

For all 47 isolates, successful amplifications were achieved across the 17 sub fragments spanning the seven MLST loci. Tables 1, 2, 3, 4, 5, 6, 7 list all SNPs included in this study. The SNP position in the fragment did not have a strong effect on the Tm separation, even if the SNP was near the amplification primer. Excluding uncA, about 90% of SNPs were transition mutations (T to C or C to T, A to G or G to A), but inversion mutations (G to C or C to G) and A to T or T to A were also readily detected.

For each sub fragment, the expected 3 to 6 alleles were resolved by HRM as distinct difference plots (Figure 1). The reproducibility

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alleles by multiple SNPs, representing both transition and which is derived from fragments representing the same MLST allele were amplified from and in replicate wells across different runs. In addition, gene extracts were run in duplicate or triplicate wells of the same plate to assign an MLST allele. For example, HRM profiles for all of the sub fragments together in order to fragment (197 bp) contained two SNPs generating three unique aspA-1 and -8 were identical as were aspA-2 and -7. The middle fragment (197 bp) contained two SNPs generating three unique sequences; but in this region, aspA-1 and -4 were identical, as were aspA-2 and -7. Finally, the right fragment (247 bp) included 3 SNPs generating 4 unique sequences, with aspA-2 and -8 being identical. Within each sub fragment, the unique sequences had distinct HRM profiles (Figure 1A, 1B, and 1C). The combination of profiles across the three sub fragments resolved the five different alleles. The uncA alleles in the demonstration set included uncA-17, which is derived from C. coli [6] and differs from the other uncA alleles by multiple SNPs, representing both transition and substitution mutations. Consequently, the HRM profile for each sub fragment of uncA-17 was highly divergent from the profiles for the other uncA alleles, with appreciably higher values for the relative signal difference (y axis, Figure 1O, P and R). For the middle sub fragment the other alleles were particularly difficult to resolve when uncA-17 was present (Figure 1P), but readily distinguished when uncA-17 was excluded (Figure 1Q). To evaluate the relative efficiency and cost of performing MLST by HRM compared with conventional direct sequencing, we analyzed a confirmation set of 84 additional C. jejuni isolates from chickens. Using HRM, we resolved 92% of the MLST alleles. Moreover, the analysis required only 35 hours of laboratory time and reagents cost only $20 (Canadian) per isolate compared with $100 for sequence-based typing (data not shown).

Discussion

MLST has emerged as the state-of-the-art method for studying bacterial population genetics. The MLST system for C. jejuni has been used in population studies of isolates from different geographical areas [17], from human and non-human sources [7,9], as well as in molecular epidemiologic analyses of outbreaks [18,19]. However performing MLST remains laborious and expensive. We have shown here that HRM can complement full MLST characterization of C. jejuni by identifying the most common alleles more rapidly and at lower cost.

HRM can resolve the SNPs that define the different alleles in the MLST system because two DNA amplicons that differ at even a single nucleotide will have different melting profiles. For the demonstration set of 47 diverse isolates, HRM resolved all 35 predicted alleles among the seven MLST genes. The differences in melting profiles among alleles varied with the number and type of SNPs as well as the gene fragment being amplified. For example, the profiles for unc-17, an allele which is known to come from C. coli [6,8,10,11,20], showed very strong differences in relative fluorescence signal and very sharp groupings (Figure 1O, P and Q). However, sub fragments where the alleles differed by only a single SNP often showed readily distinguished melting profiles (e.g., aspA-2, 7 and aspA-1, 8 in Figure 1A and aspA-7 and aspA-1 in Figure 1C). Even in instances where the relative fluorescence signal differences were quite small (0.8–3.0) and, consequently, the profiles less tightly clustered (Figure 1A, C and F), reliable interpretation was possible based on the differences in the overall profiles considered across the range of temperatures. This strategy for typing C. jejuni isolates has many important advantages, but the single greatest benefit is the reduction in the

| Table 1. SNPs in aspA locus fragments. |
|---------------------------------------|
| Allele | SNPs position (5’ to 3’) in locus fragments* |
|--------|-----------------------------------------------|
|        | 9     | 45    | 84    | 174   | 279   | 342   | 414   | 476   |
| aspA-1 | T     | G     | G     | C     | C     | T     | C     | T     |
| aspA-2 | T     | G     | A     | A     | T     | C     | C     | T     |
| aspA-4 | C     | A     | G     | G     | C     | T     | T     | C     |
| aspA-7 | T     | G     | A     | A     | T     | C     | T     | T     |
| aspA-8 | T     | G     | G     | G     | T     | C     | C     | T     |

*The numbering starts at the first nucleotide of each comparison fragment for each locus on the C. jejuni MLST database website. Numbers not underlined are in the left fragment, numbers with intermittent underlining are in the middle fragments and numbers with solid underlining are in the right fragment. doi:10.1371/journal.pone.0016167.t001

| Table 2. SNPs in gltA locus fragments. |
|---------------------------------------|
| Allele | SNPs position (5’ to 3’) in locus fragments* |
|--------|-----------------------------------------------|
|        | 12    | 39    | 200   | 201   | 207   | 294   | 320   | 348   | 396   |
| gltA-1 | A     | C     | T     | G     | C     | C     | G     | A     | A     |
| gltA-2 | G     | T     | G     | T     | G     | T     | C     | A     | A     |
| gltA-3 | A     | T     | C     | G     | C     | T     | A     | A     | G     |
| gltA-5 | A     | C     | T     | G     | C     | C     | A     | A     | A     |
| gltA-10| A     | T     | T     | C     | C     | T     | A     | A     | G     |

*The numbering starts at the first nucleotide of each comparison fragment for each locus on the C. jejuni MLST database website. Numbers not underlined are in the left fragment and numbers with solid underlining are in the right fragment. doi:10.1371/journal.pone.0016167.t002
August 2010, the of MLST since the existing nomenclature can be used. As of 2010, the HRM system provides a rapid method for the identification of pathogenic isolates. However, the standardization of the HRM system is necessary to ensure the portability of the method. HRM can be used to identify new alleles or to confirm the absence of known alleles. The HRM system is particularly efficient when analyzing ecological niches with relatively few strains. Analyzing isolates from niches with more variation, novel niches, or from several niches simultaneously would be less efficient as it would require additional reference strains or sequencing more samples, but would still be less expensive than sequencing of all genes.

Obviously, an HRM system cannot replace sequence-based MLST. If a previously unidentified melting profile is encountered, it is necessary to revert to sequencing; however, once identified, the new profile can be used for reference in subsequent HRM analyses. If the sequence proves to be a new allele, it can be submitted to the database.

### Table 4. SNPs in tkt locus fragments.

| Allele | SNPs position (5’ to 3’) in locus fragments* |
|--------|-------------------------------------------|
|        | 12 | 21 | 72 | 117 | 138 | 141 | 162 | 174 | 189 | 297 | 330 | 435 |
| tkt-1  | C  | C  | T  | C  | C  | T  | A  | A  | C  | C  | T  | C  |
| tkt-3  | C  | C  | T  | C  | C  | T  | A  | A  | C  | C  | C  | C  |
| tkt-7  | T  | T  | A  | C  | A  | C  | A  | G  | T  | T  | C  | T  |
| tkt-9  | T  | T  | A  | T  | C  | T  | G  | G  | T  | T  | C  | T  |

*The numbering starts at the first nucleotide of each comparison fragment for each locus on the C. jejuni MLST database website. Numbers not underlined are in the left fragment and numbers with solid underlining are in the right fragment.

At a technical level, HRM can be performed using machines that accept 384-well plates, permitting high-throughput studies. Because HRM compares amplicons from independent PCR reactions, it is essential to standardize the quality of DNA used in order to minimize reaction-to-reaction variability. We observed that variation in DNA quality or quality could shift amplification curves; such offsets have been previously observed to compromise the HRM groupings.

HRM can be readily applied to a wide range of genetic analyses that involve detection of a single SNP or a signature allele representing a specific set of SNPs. Examples in microbiology include studies requiring the identification of particular clonal complexes, sequence types or individual mutations. By selecting the locus amplified and the reference standard for the HRM system based on the objective of the study, this approach can be applied to questions in pathogenesis, ecology, epidemiology and antibiotic resistance. As just one example, the NAP1/027 epidemic strain of C. difficile belongs to MLST type 35. Identifying a signature allelic profile could serve as a rapid shortcut for preliminary strain detection, minimizing the challenges and effort associated with PFGE or sequencing. Analogous situations arise in numerous studies across all levels of biology, from resistance mutations in viruses to human alleles associated with clinical disease.

In summary, we have demonstrated that by analyzing multiple loci concurrently HRM technology can resolve the SNPs that are the basis of MLST. In our studies of >120 C. jejuni isolates from diverse geographical sources and representing diverse genotypes, the HRM results were consistent with sequencing and thus could be expressed using the existing MLST nomenclature, but were obtained with greater speed, less effort and at lower cost. HRM has the potential to complement classical sequence-based methods and facilitate a wide range of genotyping studies.

### Materials and Methods

#### Isolates

Table 8 lists the MLST alleles, sequence type and clonal complex of 47 C. jejuni isolates used in this study; all have been previously reported [10] and analyzed by the standard MLST protocol [7]. Isolates were selected to represent diverse sources and to include the six most prevalent sequence types (ST) and most frequent alleles for each locus.

#### DNA extraction

All C. jejuni isolates were grown on 5% (vol/vol) defibrinated sheep blood TSA (Oxoid Inc., Nepean, On) in a micro aerobic atmosphere at 42°C for 24–48 h. Isolated colonies were used to inoculate Mueller-Hinton broth (Oxoid Inc., Nepean, On), grown...
Table 6. SNPs in glyA locus fragments.

| Allele | SNPs position (5' to 3') in locus fragments* |
|--------|--------------------------------------------|
|        | 3 | 42 | 51 | 57 | 114 | 120 | 129 | 136 | 138 | 198 | 208 | 213 | 237 | 259 | 264 | 267 | 285 | 286 | 303 | 309 | 312 | 320 | 504 |
| glyA-2 | T | C | C | T | T | A | A | C | C | T | C | G | C | C | A | A | C | A | A | C | T | G | C | C |
| glyA-3 | C | T | T | C | T | A | C | C | T | T | A | T | A | G | T | G | A | T | C | T | A | C | T |
| glyA-4 | T | C | C | T | C | G | G | T | A | T | A | T | A | G | C | G | G | C | C | C | A | T | C |
| glyA-53 | T | T | T | C | C | T | T | C | T | A | T | G | G | T | G | A | T | C | T | A | C | T |

*The numbering starts at the first nucleotide of each comparison fragment for each locus on the C. jejuni MLST database website. Numbers not underlined are in the left fragment and numbers with solid underling are in the right fragment.

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to 0.5 McFarland standard density, 0.5 ml of the broth transferred to a microfuge tube, centrifuged at 13000 rpm for 10 minutes and the supernatant discarded. Genomic DNA was extracted from the pellet by adding 10 μl of NaOH 0.5 N. After 5 minutes, 10 μl of Tris 1 M pH 8.0 and 980 μl of sterile distilled water were added. DNA extracts were stored at 2°C to 0.5°C. DNA concentration was measured using a NanoVue spectrophotometer (GE Healthcare Life Science, Piscataway, NJ, USA).

Primer design

The fragments for the seven loci in the MLST system (402 to 507 bp) are longer than the maximum that can be efficiently analyzed by HRM (100 to 300 bp) [13]. Consequently, for each locus two or three sub fragments were analyzed to provide adequate resolution of the known alleles. Oligonucleotide primers used are listed in Table 9. In the majority of cases, the 3' end (for forward primers of left locus fragments) and the 5' end (for reverse primers of right locus fragments) were the last nucleotides before/after the comparison fragment for each locus on the C. jejuni MLST database website. In four cases (GLN HRM F7, TKT HRM F1, TKT HRM R2, UNC HRM F6) the primer was upstream or overlapped each other to cover the entire sequence. Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA) and used without further purification.

PCR and HRM analysis

Real-time PCR cycling was performed in a 96-well plate on a LightCycler® 480 II real-time PCR system (Roche). Each plate must contain at least two reference isolates for each allele that would be identified on the plate together with the unknown samples. The reaction was performed in a 15 µl PCR mix containing 1X LightCycler® 480 High Resolution Melting Master Kit (Roche), 3.5 mM MgCl₂, 0.5 µM of each primer and between 10 and 20 ng of DNA. The amplification protocol consisted of a first denaturation step at 95°C [5 min], 45 cycles of denaturation at 95°C [10 s], annealing at 55°C [30 s], and extension at 72°C [30 s]. The HRM step consisted of a first denaturation step at 95°C [1 min], followed by a renaturation step at 40°C [1 min]. Melting curves were generated by ramping from 70°C to 95°C at 0.02°C/sec, 25 acquisitions/°C.

During amplification, fluorescence data were normalized and then plotted using the automated grouping functionality provided by the LightCycler® 480 II Gene Scanning Software version 1.5.0.39 and by manual editing. Figure 2A shows the compilation of curves representing successful amplification of the left fragment of glyc for 96 isolates. All curves reached a similar plateau height and, as per manufacturer’s recommendations, the mean cycle number at which fluorescence exceeded background (referred to as the crossing point or cycle threshold) was <30 with a range of less than 7 across all samples. Reactions that did not meet these criteria were discarded and the fragment amplified again in a subsequent run. The software automatically analyzed the raw melting curve data and set the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to uniform values (Figure 2B); occasionally, manual adjustments were made to optimize group separation. Next, the software shifted the normalized curves along the temperature axis to equalize the point at which the dsDNA in each sample becomes completely denatured (temperature shift, Figure 2C). For each locus, the default of 5 was used as the

Table 7. SNPs in pgm locus fragments.

| Allele | SNPs position (5' to 3') in locus fragments* |
|--------|--------------------------------------------|
|        | 33 | 41 | 45 | 81 | 150 | 162 | 165 | 168 | 171 | 216 | 219 | 249 | 267 | 291 | 316 | 324 | 342 | 348 | 372 | 405 | 408 | 435 | 453 | 471 | 494 |
| pgm-1  | A | C | T | A | A | A | T | A | A | C | C | C | G | T | C | C | G | T | T | T | C | C | C |
| pgm-2  | G | T | C | G | G | G | T | A | G | G | T | G | T | T | C | C | T | A | C | C | T | T | T |
| pgm-5  | A | C | T | G | A | A | T | A | A | C | C | C | A | T | G | T | T | G | T | T | T | C | C | C |
| pgm-6  | A | C | T | G | A | G | A | C | A | C | A | C | A | T | C | T | C | T | C | C | C | T | T |
| pgm-10 | A | C | T | G | A | A | G | T | A | A | C | C | A | T | G | T | C | T | G | T | T | T | C | C |
| pgm-11 | G | T | C | G | G | G | G | G | G | A | C | G | T | T | G | T | T | C | C | T | A | C | T | T |

*The numbering starts at the first nucleotide of each comparison fragment for each locus on the C. jejuni MLST database website. Numbers not underlined are in the left fragment and numbers with solid underling are in the right fragment.

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Figure 1. Difference plots for the normalized and temperature shifted melting curves for all locus fragments. A: asp left. B: asp middle. C: asp right. D: gln left. E: gln middle. F: gln right. G: glt left. H: glt right. I: gly left. J: gly right. K: pgm left. L: pgm right. M: tkt left. N: tkt right. O: unc left. P: unc middle. Q: unc middle without allele unc-17. R: unc right. Arrows link allele numbers with corresponding same color curves. doi:10.1371/journal.pone.0016167.g001
Table 8. *C. jejuni* isolates used in the study.

| Isolate   | Source   | aspA | glnA | gltA | glyA | pgm | tkt | uncA | ST*  | CC* |
|-----------|----------|------|------|------|------|-----|-----|------|------|-----|
| 001A-0058 | Human    | 2    | 1    | 1    | 3    | 2   | 1   | 5    | 21   | 21  |
| 001A-0078 | Human    | 2    | 1    | 1    | 3    | 2   | 1   | 5    | 21   | 21  |
| 0018-0003 | Chicken  | 2    | 1    | 1    | 3    | 2   | 1   | 5    | 21   | 21  |
| 0018-0035 | Chicken  | 2    | 1    | 1    | 3    | 2   | 1   | 5    | 21   | 21  |
| 0018-0046 | Chicken  | 2    | 1    | 1    | 3    | 2   | 1   | 5    | 21   | 21  |
| 006A-0001 | Raw milk | 2    | 1    | 1    | 3    | 3   | 1   | 5    | 21   | 21  |
| 006A-0004 | Raw milk | 2    | 1    | 1    | 3    | 2   | 1   | 5    | 21   | 21  |
| 007A-0018 | Water    | 2    | 1    | 1    | 3    | 2   | 1   | 5    | 21   | 21  |
| 007A-0031 | Water    | 2    | 1    | 1    | 3    | 2   | 1   | 5    | 21   | 21  |
| 001A-0059 | Human    | 4    | 7    | 10   | 4    | 1   | 7   | 1    | 45   | 45  |
| 001A-0060 | Human    | 4    | 7    | 10   | 4    | 1   | 7   | 1    | 45   | 45  |
| 0018-0010 | Chicken  | 4    | 7    | 10   | 4    | 1   | 7   | 1    | 45   | 45  |
| 0018-0011 | Chicken  | 4    | 7    | 10   | 4    | 1   | 7   | 1    | 45   | 45  |
| 0018-0024 | Chicken  | 4    | 7    | 10   | 4    | 1   | 7   | 1    | 45   | 45  |
| 007A-0023 | Water    | 4    | 7    | 10   | 4    | 1   | 7   | 1    | 45   | 45  |
| 007A-0030 | Water    | 4    | 7    | 10   | 4    | 1   | 7   | 1    | 45   | 45  |
| 007A-0032 | Water    | 4    | 7    | 10   | 4    | 1   | 7   | 1    | 45   | 45  |
| 001A-0005 | Human    | 7    | 17   | 5    | 2    | 10  | 3   | 6    | 353  | 353 |
| 001A-0016 | Human    | 7    | 17   | 5    | 2    | 10  | 3   | 6    | 353  | 353 |
| 001A-0085 | Human    | 7    | 17   | 5    | 2    | 10  | 3   | 6    | 353  | 353 |
| 001A-0259 | Human    | 7    | 17   | 5    | 2    | 10  | 3   | 6    | 353  | 353 |
| 001A-0263 | Human    | 7    | 17   | 5    | 2    | 10  | 3   | 6    | 353  | 353 |
| 001A-0273 | Human    | 7    | 17   | 5    | 2    | 10  | 3   | 6    | 353  | 353 |
| 001A-0274 | Human    | 7    | 17   | 5    | 2    | 10  | 3   | 6    | 353  | 353 |
| 0018-0008 | Chicken  | 7    | 17   | 5    | 2    | 10  | 3   | 6    | 353  | 353 |
| 001A-0162 | Human    | 1    | 4    | 2    | 2    | 6   | 3   | 17   | 61   | 61  |
| 001A-0163 | Human    | 1    | 4    | 2    | 2    | 6   | 3   | 17   | 61   | 61  |
| 001A-0166 | Human    | 1    | 4    | 2    | 2    | 6   | 3   | 17   | 61   | 61  |
| 001A-0238 | Human    | 1    | 4    | 2    | 2    | 6   | 3   | 17   | 61   | 61  |
| 006A-0014 | Raw milk | 1    | 4    | 2    | 2    | 6   | 3   | 17   | 61   | 61  |
| 006A-0020 | Raw milk | 1    | 4    | 2    | 2    | 6   | 3   | 17   | 61   | 61  |
| 006A-0026 | Raw milk | 1    | 4    | 2    | 2    | 6   | 3   | 17   | 61   | 61  |
| 006A-0028 | Raw milk | 1    | 4    | 2    | 2    | 6   | 3   | 17   | 61   | 61  |
| 001A-0064 | Human    | 1    | 2    | 3    | 4    | 5   | 9   | 3    | 42   | 42  |
| 001A-0084 | Human    | 1    | 2    | 3    | 4    | 5   | 9   | 3    | 42   | 42  |
| 001A-0088 | Human    | 1    | 2    | 3    | 4    | 5   | 9   | 3    | 42   | 42  |
| 001A-0168 | Human    | 1    | 2    | 3    | 4    | 5   | 9   | 3    | 42   | 42  |
| 001B-0009 | Chicken  | 1    | 2    | 3    | 4    | 5   | 9   | 3    | 42   | 42  |
| 001B-0012 | Chicken  | 1    | 2    | 3    | 4    | 5   | 9   | 3    | 42   | 42  |
| 001B-0052 | Chicken  | 1    | 2    | 3    | 4    | 5   | 9   | 3    | 42   | 42  |
| 006A-0053 | Raw milk | 1    | 2    | 3    | 4    | 5   | 9   | 3    | 42   | 42  |
| 001A-0287 | Human    | 8    | 2    | 5    | 53   | 11  | 3   | 105  | 1212 | 1212 |
| 001A-0289 | Human    | 8    | 2    | 5    | 53   | 11  | 3   | 105  | 1212 | 1212 |
| 001B-0029 | Chicken  | 8    | 2    | 5    | 53   | 11  | 3   | 105  | 1212 | 1212 |
| 001B-0055 | Chicken  | 8    | 2    | 5    | 53   | 11  | 3   | 105  | 1212 | 1212 |
| 001B-0056 | Chicken  | 8    | 2    | 5    | 53   | 11  | 3   | 105  | 1212 | 1212 |
| 001B-0057 | Chicken  | 8    | 2    | 5    | 53   | 11  | 3   | 105  | 1212 | 1212 |

*ST; sequence type.

CC; clonal complex.

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Table 9. Oligonucleotide primers used in the study.

| Locus | Locus fragment | Forward (5' to 3') | Reverse (5' to 3') | Amplicon size (bp) |
|-------|----------------|--------------------|-------------------|--------------------|
| aspA  | asp left       | ASP HRM F3         | GTG AAT TTA AAA CTT TTG CGA TA | ASP HRM R6         | TCG ATC AAA TCC TCA GCC ACA GTA | 199 |
|       | asp middle     | ASP HRM F5         | TAA GAG AAG TGA CAG GTT TTG AAT | ASP HRM R7         | GGA AGA TTA ATC TCA TTA AGA CCA CAT T | 187 |
|       | asp right      | ASP HRM F7         | GAC TTA AGA CTT TTA AGT AGT GGT CC | ASP HRM R4         | GCA TTA CAA CAG AAT TAA ATA AGC TAT ATG C | 247 |
| glnA  | gln left       | GLN HRM F6         | AAC CTT ATG CTC AAA GTG C | GLN HRM R5         | CAT TTT TCA TAC ATT TGT CCT TTG | 106 |
|       | gln middle     | GLN HRM F7         | CTA TCA TAG TAT TTG TTG ATG TGT ATG | GLN HRM R4         | CTA AAG AAT CAA TTG GCT GAA GTG G | 318 |
|       | gln right      | GLN HRM F4         | CTG GAC ACA GGC CAA GAA ACA AAG GTG | GLN HRM R2         | GAG CTA CCA TTT TTA CAA CAT ATT TAT AAA TTT G | 231 |
| gltA  | glt left       | GLT HRM F1         | CCG GTC TTG AAG CAT TTC GGT AT | GLT HRM R1         | CCA CTA TAG GGA TTT TAG CTA C | 225 |
|       | glt right      | GLT HRM F2         | GAA TAT ATG GAA ATG GCA GCT AG | GLT HRM R2         | GCA TGA GTT GAA CCC ACA GC | 272 |
| glyA  | gly left       | GLY HRM F3         | GAT AAA ATT TTA GGA ATG GAT TTA AGT CAT G | GLY HRM R1         | CAC AAC AAG ACC TGG AAT ATG | 288 |
|       | gly right      | GLY HRM F2         | GCC TAT CTT TTT GGT ATG GTC ACG C | GLY HRM R2         | AAA ACA TTA GCT AAA ACT TGA GC | 317 |
| pgm   | pgm left       | PGM HRM F1         | GAA GTT ATA GTA AGT GAT GAA AAA CCT ATG A | PGM HRM R1         | TTT AAA GCA CCA TTA CTC ATT ATA GT | 275 |
|       | pgm right      | PGM HRM F4         | GAA AAA TTA CAA TCA AGT GGT GCT GC | PGM HRM R3         | CTT TTT TTT CTG CAA TTT TAA G | 328 |
| tkt   | tkt left       | TKT HRM F1         | CAT GCA AGT GGT TTG CTT TAT AGT | TKT HRM R1         | CCC ATC TCC CCA AAG ACA A | 261 |
|       | tkt right      | TKT HRM F2         | GCT AGG CAG TGA TTT AAT CGA TCA | TKT HRM R2         | GAT GAT AAG ACA AGG TTT TGT GGA | 304 |
| uncA  | unc left       | UNC HRM F6         | GGT GCT ATG GAA TAT ACT ATT GGT G | UNC HRM R3         | GAC ATT TCG CGA TAA GCT ACA GC | 176 |
|       | unc middle     | UNC HRM F7         | GGT TAT GAT GTT AGT ACG AAG C | UNC HRM R4         | GGT GGA ATA TAA GAA GAA ACA GCC TCT CC | 221 |
|       | unc right      | UNC HRM F8         | GGT GCT GGT TTG TTG AGC GCA TTG | UNC HRM R2         | GGT CAA AAG CTG GAA GCT CCT TA | 265 |
Amplification Curves

Fluorescence (465-510)

Cycles

Melting Curves

Fluorescence (465-510)

Temperature [°C]

Normalized and Shifted Melting Curves

Relative Signal [%]

Temperature [°C]
threshold value in the temperature shift step. In the final step each shifted, normalized curve is plotted (difference plot, Figure 1I) as the difference relative to an arbitrarily chosen reference curve. Curves not grouped with one of the reference isolates would have to be run subsequently with other reference isolates containing the allele or sequenced. If the reference isolates were not grouped together correctly, the run would be repeated.

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Author Contributions

Conceived and designed the experiments: SL EHF. Performed the experiments: SL. Analyzed the data: SL SM RDA EHF. Contributed reagents/materials/analysis tools: SM EF. Wrote the paper: SL SM RDA EHF.

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