MS-H: A Novel Proteomic Approach to Isolate and Type the E. coli H Antigen Using Membrane Filtration and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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Abstract

Serotyping is the long-standing gold standard method to determine E. coli H antigens; however, this method requires a panel of H-antigen specific antibodies and often culture-based induction of the H-antigen flagellar motility. In this study, a rapid and accurate method to isolate and identify the Escherichia coli (E. coli) H flagellar antigen was developed using membrane filtration and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Flagella were isolated from pure culture, digested with trypsin, and then subjected to LC-MS/MS using one of two systems (Agilent-nano-LC-QSTAR XL or Proxeon-nano-LC-LTQ-Orbitrap XL). The resulting peptide sequence data were searched against a custom E. coli flagella/H antigen database. This approach was evaluated using flagella isolated from reference E. coli strains representing all 53 known H antigen types and 41 clinical E. coli strains. The resulting LC-MS/MS classifications of H antigen types (MS-H) were concordant with the known H serogroup for all 53 reference types, and of 41 clinical isolates tested, 38 (92.7%) were concordant with the known H serogroup. MS-H clearly also identified two clinical isolates (4.9%) that were untypeable by serotyping. Notably, successful detection and classification of flagellar antigens with MS-H did not generally require induction of motility, establishing this proteomic approach as more rapid and cost-effective than traditional methods, while providing equitable specificity for typing E. coli H antigens.

Introduction

Traditional typing methods of E. coli bacteria involve biochemical tests and serotyping of O antigens (lipopolysaccharides) on the bacterial surface, K antigens from the capsule, and H antigens on the extracellular flagella [1]. Serotyping of the H antigen involves the examination of 53 distinct types of flagella (H1 to H56; designations H13, H22, and H50 no longer exist [2]), and is commonly used to identify and classify clinical and food-borne isolates of E. coli, with notable classifications including the most commonly seen O157:H7 group and the “non-O157” group representing other toxigenic strains [3]. However, conventional serotyping methodology based on antisera can be costly and laborious to perform due to varying quality of antibody preparations and the number of antibody agglutination reactions needed to assign a final classification [4,5]. When bacterial cells do not generate lipopolysaccharide on the surface, the cultured colonies become “rough strains”, and both O and H antigen identification by antibody-based agglutination may be problematic despite the retention of cellular motility and presence of the H antigen flagellar structure [1,5].

Molecular typing methods using polymerase chain reaction (PCR)-based amplification and targeted genetic sequencing are gaining popularity as a means for serotype classification due to their potential for higher throughput [4–6]. Other recent technologies for bacterial classification and identification include the application of mass spectrometry (MS) for bacterial nucleic acid detection, mass pattern analysis [7], and the quantification of bacterial proteins [8]. Matrix-associated laser-desorption/ionization time-of-flight (MALDI-TOF) MS usage for whole bacterial protein profiling to classify and type bacteria has also shown some promising results due to the ease of use and high throughput potential [9,10].

Flagella are homopolymeric filaments comprising 40–60 kDa flagellin subunits, with E. coli flagellum filaments approximately 15–20 μm long and 20 nm in diameter [11,12]. Flagella have roles in bacterial motility, adhesion to substrates, biofilm formation, and virulence processes [12]. When studied in vitro, flagella are easily sheared off the bacterial surface by physical
forces such as vortexing or thin-needle shearing, and can be purified by ultracentrifugation [11]. They are also heat-labile and easily digested into peptides at 37°C [12].

In this study, a method to rapidly determine E. coli H antigen types was developed, which combines the isolation of flagella on a filter membrane followed by enzyme digestion and online LC-MS/MS of the flagellin peptides using one of two LC-MS/MS platforms: Agilent-nano-LC-QSTAR XL (QSTAR in brief) or Proxeon-nano-LC-LTQ-Orbitrap XL (Orbitrap in brief). Comparing the resulting peptide sequence data to a custom reference H antigen protein database allowed for classification of H antigen types. When compared to traditional serotyping, this proteomic approach for E. coli flagellar H typing through MS, described here as MS-H, was found to be equally specific, but also a more rapid and reproducible means of obtaining H antigen type information without the requirement of antisera and motility induction.

Results

Method development: Proof-of-principle using H7

Detailed procedures describing flagella purification, enzymatic digestion, and sample preparation for LC-MS/MS are described in the Materials and Methods. In brief, flagella were detached from their bacterial walls by vortexing a liquid E. coli cell suspension after overnight culture on agar. High speed centrifugation was then used to separate the flagella (in the supernatant) from the cellular pellet [12,13]. Flagella were isolated on a membrane syringe filter which additionally provided an optimum substrate for rapid buffer exchange, minimal contamination, and efficient on-membrane tryptic digestion [14]. The digest was flushed out of the syringe filter, vacuum dried, and applied onto QSTAR for MS-H.

A curated database of reference flagellin proteins was established to enable the final classification of H types from peptide sequences deduced by LC-MS/MS and a Mascot search engine. This database included all available E. coli flagellin protein sequences from NCBI, with each sequence denoted by its known H antigen serogroup (Figure S1). Using this custom database and the Mascot search engine, the identity and classification of H antigen serogroups from flagellin peptide data was determined by using a minimum of two serogroup-specific peptide sequences [15]. MS-H types were assigned as the top scoring hit in the identified protein list possessing the highest confidence score. If more than one H type represented the top scoring hit, the result would be considered ambiguous.

To determine the method’s ability to rapidly isolate flagella and classify H antigen serogroups after LC-MS/MS, 11 E. coli reference isolates known to express the H7 antigen and one known non-motile E. coli reference isolate (E32511) were tested. Using a minimum of two serogroup-specific peptide sequences [15]. MS-H types were assigned as the top scoring hit in the identified protein list possessing the highest confidence score. If more than one H type represented the top scoring hit, the result would be considered ambiguous.

To determine the method’s ability to rapidly isolate flagella and classify H antigen serogroups after LC-MS/MS, 11 E. coli reference isolates known to express the H7 antigen and one known non-motile E. coli reference isolate (E32511) were tested. Duplicate experiments were performed on E. coli strains cultured from frozen stocks without any induction of motility. For each of the H7 isolates, a minimum of 60% peptide sequence coverage was obtained based on the reference H7 protein sequences included in the curated database for all of the E. coli flagellin protein sequences, and in all instances H7 was the top-scoring hit, indicating 100% specificity. For the known non-motile strain E32511, there were no matches to flagellin peptides (Table 1).

Analytical sensitivity [16,17] was determined by diluting the flagellin digest (dilution factor ranged from 2 to 100) of one reference strain (87-1215). Since a major component of the syringe filter digests was the added trypsin, a parallel experiment was done to purify the intact flagella by ultracentrifugation of the flagella-containing supernatant [18], after which protein quantitation was carried out on the intact flagellin. This flagellin identification method was found to be very sensitive, as good sequence coverage and an accurate identification of H7 antigen was achieved with the QSTAR using a sub-microgram detection level of flagella. In general, a higher flagella concentration yielded higher protein sequence coverage (Table S1).

Advanced evaluation of specificity by testing the full panel of H antigen types

MS-H typing of all E. coli H antigens was completed using bacterial stocks of reference strains. All 53 types were successfully identified from overnight cultures of frozen stocks without motility induction (Table 2). For strains that lost flagellar motility, confirmed by subsequent electron microscopy (EM) observations and motility tests, alternate reference strains having the same H types were selected for analyses. This examination also confirmed that the curated database was suitable for specific identification of all H types. Detection of flagellin by EM (Figure S2) and characterization of intact flagellin by SDS-PAGE (Figure S3) showed that the production of flagella and the expression of flagellin were quite heterogeneous.

A comparison of MS-H and serotyping was then performed on 41 clinical isolates randomly chosen over a three-month period from incoming E. coli samples for routine serotyping. 38 samples gave identical results for both MS-H and the traditional serotyping method (Table 3). However, strain 09-0417, which was H7 by serotyping and then became untypeable, and strain 09-1760, which was also untypeable, were confirmed to be H21 by MS-H and DNA sequencing (Table S2). Strain 09-1775 (serotype H25), an unstable strain that became rough during the serotyping process, exhibited low sequence coverage for MS-H (H4, coverage 8%, Table 3). This isolate was confirmed to be MS-H 25 later by the more sensitive Orbitrap system for side-by-side comparison of MS-H typing and serotyping. In summary, 92.7% (i.e. 38 of 41

### Table 1. H7 identification of reference strains by MS-H with the QSTAR platform

| Strain number | MS-H type | Sequence coverage (%) |
|---------------|-----------|-----------------------|
| EDL-933       | H7        | 72                    | 68                |
| E175          | H7        | 72                    | 86                |
| 06-1139       | H7        | 79                    | 76                |
| 06-3122       | H7        | 80                    | 90                |
| 07-0909       | H7        | 67                    | 61                |
| 07-0918       | H7        | 61                    | 74                |
| 07-1591       | H7        | 78                    | 80                |
| 07-1756       | H7        | 75                    | 88                |
| 07-1946       | H7        | 75                    | 88                |
| 87-1215       | H7        | 82                    | 87                |
| 90-2380       | H7        | 71                    | 78                |
| E32511 (non-motile) | No flagellin detected | 0 | 0 |

*11 known H7 positive E. coli strains and one non-motile strain (E32511) were twice tested for MS-H, and the sequence coverage were obtained by Mascot database search.

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E. coli H Typing by Mass Spectrometry
Table 2. MS-H identification of all references strains with the QSTAR platforma.

| Recorded H serotype | Representative strain | MS-H types with no motility induced | MS-H type sequence coverage (%) | Number of strains tested by MS-H |
|---------------------|-----------------------|-------------------------------------|---------------------------------|---------------------------------|
| H1                  | E169                  | H1                                  | 61                              | 1                               |
| H2                  | E170                  | H2                                  | 81                              | 2 (1 UI)                        |
| H3                  | E171                  | H3                                  | 49                              | 1                               |
| H4                  | E172                  | H4                                  | 88                              | 1                               |
| H5                  | E173                  | H5                                  | 45                              | 1                               |
| H6                  | E174                  | H6                                  | 65                              | 1                               |
| H7                  | E175                  | H7                                  | 68                              | 1                               |
| H8                  | E176                  | H8                                  | 78                              | 1                               |
| H9                  | E177                  | H9                                  | 74                              | 1                               |
| H10                 | E659                  | H10                                 | 62                              | 2 (1 UI)                        |
| H11                 | 07-6285               | H11                                 | 70                              | 2 (1 UI)                        |
| H12                 | E241                  | H12                                 | 47                              | 1                               |
| H14                 | E182                  | H14                                 | 89                              | 1                               |
| H15                 | E183                  | H15                                 | 93                              | 1                               |
| H16                 | E184                  | H16                                 | 81                              | 1                               |
| H17                 | E185                  | H17                                 | 87                              | 1                               |
| H18                 | E186                  | H18                                 | 30                              | 3 (1 UI)                        |
| H19                 | 09-0523               | H19                                 | 69                              | 2                               |
| H20                 | E188                  | H20                                 | 94                              | 1                               |
| H21                 | E189                  | H21                                 | 71                              | 1                               |
| H23                 | E191                  | H23                                 | 69                              | 1                               |
| H24                 | E192                  | H24                                 | 88                              | 1                               |
| H25                 | E193                  | H25                                 | 86                              | 1                               |
| H26                 | E194                  | H26                                 | 95                              | 1                               |
| H27                 | E195                  | H27                                 | 76                              | 1                               |
| H28                 | E196                  | H28                                 | 49                              | 1                               |
| H29                 | E197                  | H29                                 | 98                              | 1                               |
| H30                 | E198                  | H30                                 | 58                              | 1                               |
| H31                 | E199                  | H31                                 | 49                              | 1                               |
| H32                 | E200                  | H32                                 | 70                              | 3 (1 UI)                        |
| H33                 | E201                  | H33                                 | 70                              | 1                               |
| H34                 | E589                  | H34                                 | 62                              | 1                               |
| H35                 | E203                  | H35                                 | 58                              | 2                               |
| H36                 | E204                  | H36                                 | 89                              | 1                               |
| H37                 | E205                  | H37                                 | 81                              | 1                               |
| H38                 | E206                  | H38                                 | 99                              | 1                               |
| H39                 | E207                  | H39                                 | 81                              | 1                               |
| H40                 | E208                  | H40                                 | 95                              | 1                               |
| H41                 | E209                  | H41                                 | 79                              | 1                               |
| H42                 | E210                  | H42                                 | 29                              | 2                               |
| H43                 | E211                  | H43                                 | 83                              | 1                               |
| H44                 | E212                  | H44                                 | 87                              | 1                               |
| H45                 | E213                  | H45                                 | 47                              | 1                               |
| H46                 | E214                  | H46                                 | 37                              | 1                               |
| H47                 | E346                  | H47                                 | 58                              | 1                               |
| H48                 | E247                  | H48                                 | 70                              | 1                               |
| H49                 | E248                  | H49                                 | 79                              | 1                               |
| H51                 | E372                  | H51                                 | 43                              | 1                               |
| H52                 | E373                  | H52                                 | 47                              | 1                               |

A. H typing by Mass Spectrometry
isolates) of the MS-H results matched the corresponding serotyping result, with two untypeable strains (i.e. 2 of 41 isolates, 4.9%) by serotyping being clearly assigned to unique H types by MS-H. Notably, the traditional serotyping analysis may have been inconsistent due to the possibility of isolates changing their phenotypes from smooth to rough during the cell culture and motility induction processes.

A side-by-side comparison of MS-H typing and serotyping

During the initial method development and advanced evaluation stages, MS-H identification of *E. coli* H types was compared to previous independent serotyping results (“gold standards”) with stock isolates. However, *E. coli* strains can be quite heterogeneous and dynamic in terms of flagella growth and flagellin production as shown above. For example, previously identified motile reference strains may not produce flagella from their frozen stocks, and some clinical strains may become rough during the subculture and motility induction processes. A side-by-side comparison was therefore required to further evaluate MS-H typing with serotyping. Four reference strains of known H type were randomly selected for side-by-side testing of motility, serotyping, and MS-H on the QSTAR consecutively for 16 days. After 24 hours of culturing, only one strain could be typed by traditional serotyping, but MS-H was able to identify three. On day 7, both serotyping and MS-H could identify three of the four strains, while strain E-375 (H55) turned rough and was no longer typeable by the traditional method. On day 16 of motility induction, E-375 remained rough, but could be identified as MS-H 55 using flagellin extracted from the motility-induced culture (Table S3). This indicates that MS-H can be successfully performed on rough strains after motility induction.

A side-by-side comparison between serotyping and MS-H for *E. coli* flagella identification was then expanded to 12 previously-typed H7 strains and other reference strains encompassing all 53 H types using the Orbitrap platform. The repeatability of MS-H identification was also tested by performing three tests on each strain. Serotyping was arranged to be done in parallel with MS-H two years earlier with the QSTAR (Table 2).

Table 2. Cont.

| Recorded H serotype | Representative strain | MS-H types with no motility induced | Number of strains tested by MS-H |
|---------------------|-----------------------|-------------------------------------|----------------------------------|
| HS3                 | E374                  | HS3 74                              | 1                                |
| HS4                 | E377                  | HS4 76                              | 1                                |
| HS5                 | E375                  | HS5 74                              | 1                                |
| HS6                 | E376                  | HS6 46                              | 1                                |

UI, unidentified strain.

*Known reference strains encompassing all 53 H types were tested by MS-H. If a primary strain could not be identified after three consecutive MS-H analyses, an alternate strain was selected for MS-H typing.

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Diagnostic sensitivity and specificity, run-to-run repeatability and instrument-to-instrument reproducibility were also tested on the Orbitrap platform using the earlier clinical strains and the residual flagellin digests from earlier method evaluation on the QSTAR. MS-H reaches 100% diagnostic specificity and 100% diagnostic sensitivity with the Orbitrap platform. In addition, the Orbitrap instrument gave consistent results when runs were performed in triplicate on the same sample for each strain. Repeated runs on residual digests, which were frozen at -80°C for two years prior to testing, showed excellent sample stability and reproducibility, and the current instrumentation gave much better sequence coverage for MS-H with less sample loading (Table 5).

Detection limit tests were designed to determine the smallest amount of culture needed for flagella extraction, and the highest dilution of digested flagellin needed for MS-H. Reference strain 87-1215 (O157:H7) was used for this experiment. Colonies from the subculture of a single colony were counted after serial dilutions and the colony count average was used to calculate cell numbers within the single colony. The culture collection size of 2.16×10^14 cells from 500 colonies was very similar to a full 10 µl loop size routinely used for flagella extraction. However, since the absolute amount of flagellin could not be quantified due to trypsin contamination during digestion, the fraction of the total digest was then used as the amount of sample loaded on to the nano-LC column. The test shows that 1/100 loopful of cell culture collection (i.e. 2×10^12 cells from 5 colonies) could still give accurate identification for MS-H, and the use of 500 colonies gave the best sequence coverage for MS-H using only 1/160 of the flagellin digest (Table S4).
**Table 3.** Comparison of \( H \) serotyping and MS-H typing results for clinical isolates with the QSTAR platform*.

| Strain number | Motility | Serotypes with motility induced | MS-H (without motility induction) / sequence coverage |
|---------------|----------|--------------------------------|--------------------------------------------------|
|               |          |                                | H types                                   | Sequence coverage (%) |
| 09-0409       | M        | H28                            | H28                                     | 50                    |
| 09-0410       | M        | H28                            | H28                                     | 40                    |
| 09-0411       | M        | H11                            | H11                                     | 58                    |
| 09-0412       | M        | H49                            | H49                                     | 42                    |
| 09-0413       | M        | H16                            | H16                                     | 53                    |
| 09-0414       | M        | H7                             | H7                                     | 51                    |
| 09-0415       | M        | H8                             | H8                                     | 38                    |
| 09-0416       | M        | H28                            | H28                                     | 44                    |
| 09-0417       | M        | H7, then untypeable            | H21\(^{b}\)                               | 80                    |
| 09-1340       | M        | H11                            | H11                                     | 52                    |
| 09-1341       | M        | H11                            | H11                                     | 63                    |
| 09-1342       | M        | H11                            | H11                                     | 57                    |
| 09-1343       | M        | H11                            | H11                                     | 50                    |
| 09-1344       | M        | H11                            | H11                                     | 52                    |
| 09-1347       | M        | H19                            | H19                                     | 56                    |
| 09-1348       | M        | H19                            | H19                                     | 41                    |
| 09-1349       | M        | H7                             | H7                                     | 39                    |
| 09-1350       | M        | H7                             | H7                                     | 45                    |
| 09-1351       | M        | H7                             | H7                                     | 41                    |
| 09-1352       | M        | H7                             | H7                                     | 39                    |
| 09-1353       | M        | H25                            | H25                                     | 42                    |
| 09-1354       | M        | H25                            | H25                                     | 28                    |
| 09-1760       | M        | untypeable                     | H21\(^{b}\)                               | 71                    |
| 09-1764       | M        | H19                            | H19                                     | 41                    |
| 09-1765       | M        | H11                            | H11                                     | 77                    |
| 09-1766       | M        | H34                            | H34                                     | 14                    |
| 09-1767       | M        | H11                            | H11                                     | 72                    |
| 09-1768       | M        | H14                            | H14                                     | 40                    |
| 09-1769       | M        | H14                            | H14                                     | 65                    |
| 09-1770       | M        | H19                            | H19                                     | 45                    |
| 09-1774       | M        | H19                            | H19                                     | 49                    |
| 09-1775       | M        | H25, then rough               | H4                                      | 8                     |
| 09-2554       | M        | H21                            | H21                                     | 73                    |
| 09-2555       | M        | H21                            | H21                                     | 72                    |
| 09-2560       | M        | H21                            | H21                                     | 83                    |
| 09-1336       | NM       | UI                             | UI                                      |                       |
| 09-1337       | NM       | UI                             | UI                                      |                       |
| 09-1338       | NM       | UI                             | UI                                      |                       |
| 09-1339       | NM       | UI                             | UI                                      |                       |
| 09-1345       | NM       | UI                             | UI                                      |                       |
| 09-1346       | NM       | UI                             | UI                                      |                       |

*Incoming clinical \( \text{E. coli} \) samples, collected over a three-month period for routine serotyping, were selected and MS-H was performed independently without motility induction; \(^{b}\)PCR-based DNA sequencing carried out for confirmation of H21.

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### Table 4. Side-by-side comparison of H serotyping and MS-H typing of *E. coli* H types with the Orbitrap platform.

| Strain Number | Previously recorded H-types with induced motility | Serotyping without induced motility | MS-H | Sequence coverage (%) |
|---------------|-----------------------------------------------|-----------------------------------|------|---------------------|
|               |                                               |                                   |      | Day 1 | Day 2 | Day 3 |
| 06-4319       | H7                                            | H7                               | H7   | 54    | 62    | 87    |
| 06-1139       | H7                                            | –                                | H7   | 87    | 84    | 87    |
| 07-1591       | H7                                            | –                                | H7   | 93    | 89    | 81    |
| 07-1756       | H7                                            | –                                | H7   | 85    | 87    | 87    |
| EDL933        | H7                                            | H7                               | H7   | 90    | 71    | 88    |
| 90-2380       | H7                                            | –                                | H7   | 85    | 83    | 90    |
| 05-0958       | H7                                            | –                                | H7   | 81    | 62    | 88    |
| 09-0414       | H7                                            | –                                | H7   | 94    | 79    | 89    |
| 09-1349       | H7                                            | –                                | H7   | 94    | 88    | 90    |
| 09-1350       | H7                                            | –                                | H7   | 91    | 73    | 90    |
| 09-1351       | H7                                            | –                                | H7   | 77    | 72    | 88    |
| 09-1352       | H7                                            | –                                | H7   | 90    | 83    | 88    |
| E169          | H1                                            | H1                               | H1   | 98    | 83    | 83    |
| E170          | H2                                            | H2                               | H2   | 67    | 71    | 68    |
| E171          | H3                                            | –                                | H3   | 92    | 90    | 86    |
| E172          | H4                                            | H4                               | H4   | 89    | 99    | 88    |
| E173          | H5                                            | –                                | H5   | 81    | 76    | 58    |
| E174          | H6                                            | H6                               | H6   | 90    | 80    | 63    |
| E176          | H8                                            | –                                | H8   | 90    | 90    | 77    |
| E177          | H9                                            | H9                               | H9   | 80    | 77    | 80    |
| E659          | H10                                           | –                                | H10  | 99    | 79    | 85    |
| 07-6285       | H11                                           | –                                | H11  | 85    | 84    | 80    |
| E241          | H12                                           | –                                | –    | –     | –     | –     |
| E241M         | H12                                           | H12                             | H12  | 98    | 97    | 97    |
| E752          | H12                                           | –                                | H12  | 98    | 91    | 97    |
| E182          | H14                                           | –                                | H14  | 47    | 81    | 55    |
| E183          | H15                                           | H15                             | H15  | 73    | 81    | 79    |
| E184          | H16                                           | –                                | H16  | 64    | 81    | 77    |
| E185          | H17                                           | –                                | H17  | 74    | 85    | 84    |
| E186          | H18                                           | H18                             | H18  | 58    | 68    | 65    |
| 09-0523       | H19                                           | H19                             | H19  | 66    | 89    | 88    |
| E188          | H20                                           | H20                             | H20  | 81    | 73    | 81    |
| E189          | H21                                           | H21                             | H21  | 95    | 98    | 95    |
| E191          | H23                                           | H23                             | H23  | 65    | 67    | 79    |
| E192          | H24                                           | H24                             | H24  | 72    | 65    | 74    |
| E193          | H25                                           | H25                             | H25  | 66    | 73    | 68    |
| E194          | H26                                           | H26                             | H26  | 98    | 75    | 85    |
| E195          | H27                                           | H27                             | H27  | 71    | 63    | 69    |
| E196          | H28                                           | –                                | H28  | 70    | 73    | 79    |
| E197          | H29                                           | H29                             | H29  | 83    | 87    | 87    |
| E198          | H30                                           | –                                | H30  | 78    | 78    | 79    |
| E199          | H31                                           | –                                | H31  | 59    | 40    | 76    |
| E200          | H32                                           | H32                             | H32  | 72    | 42    | 67    |
| E201          | H33                                           | –                                | H33  | 70    | 65    | 75    |
| E589          | H34                                           | H34                             | H34  | 65    | 66    | 67    |
| E203          | H35                                           | –                                | H35  | 51    | 56    | 45    |
| E204          | H36                                           | rough                          | H36  | 90    | 90    | 92    |
Discussion

This study of LC-MS/MS-based method development and evaluation of *E. coli* H typing (MS-H) was based on international analytical method validation guidelines as they pertain to the characteristics of current *E. coli* serotyping for clinical diagnosis [16,20]. The flagella purification assay was modified from traditional flagella purification procedures [13], but omitted tedious ultracentrifugation and gradient separation of the large volume of cell culture. Further, the process was specific for flagella due to their unique polymerized structure, size, and length [11–13]. The methodology not only made sample preparation faster and easier, but also minimized the presence of MS intolerable residues [14].

A 10 μl loopful of culture grown on TSA agar was sufficient to extract flagella on a 13 mm diameter filter for MS-H. Since flagella extraction and trypsic digestion were limited to a tiny, fixed space (roughly 80 μl) of syringe filter, more flagellin products relatively reduced the ratio of trypsin used in the digest, giving a much stronger flagellin to trypsin MS signal. Consequently, it is recommended to use an almost-full 10 μl loopful of fresh bacterial culture in order to achieve less noise-interference from trypsin auto-digestion. The QSTAR system gave valid results after loading half (i.e. 10 μl of 20 μl) of the re-dissolved flagellin preparation following vacuum drying of the digest. With the Orbitrap platform, accurate results were obtained with only 1/120 (i.e. 5 μl of 600 μl) of the digest without the need for vacuum drying. Additionally, the quantity of digested flagella was far beyond the need [15] for protein identification using this system, with more than 50% protein sequence coverage routinely obtained from a small fraction of the flagella digest on Orbitrap platform. This may be attributed to the purity of the flagella through such unique extraction and digestion methods, which also enabled the differentiation of H types with close sequence similarity. Sample analyses of LC-MS/MS with the two instrumentation platforms (QSTAR, Orbitrap) used in this study have proven that MS-H is reproducible and robust.

While embarking on database searches at the onset of this project, it was discovered that public databases such as NCBIinr or Swiss-prot do not always display the necessary information needed for H antigen type investigation, and in some cases, there is no H type specified for the flagellin protein. Thus, a custom flagellin database was generated with the H type listed in the flagellin protein description. This curated database proved useful in obtaining correct MS-H types, and is available in the supporting information (Protein Database S1).

Table 4 summarizes the features of both MS-H and traditional serotyping. From this study, it can be concluded that the two methods possess similar diagnostic sensitivity and specificity.

Table 4. Cont.

| Strain Number | Previously recorded H-types with induced motility | Serotyping without induced motility | MS-H | Sequence coverage (%) |
|---------------|-----------------------------------------------|-----------------------------------|------|-----------------------|
| E205          | H37                                           | –                                 | H37  | 96 91 85              |
| E206          | H38                                           | H38                               | H38  | 97 98 86              |
| E207          | H39                                           | H39                               | H39  | 83 68 61              |
| E208          | H40                                           | H40                               | H40  | 97 92 91              |
| E209          | H41                                           | –                                 | H41  | 72 47 50              |
| E210          | H42                                           | –                                 | H42  | 48 – 36               |
| E210M*        | H42                                           | H42                               | H42  | 72 70 74              |
| E211          | H43                                           | –                                 | H43  | 90 89 89              |
| E212          | H44                                           | H44                               | H44  | 78 74 76              |
| E213          | H45                                           | –                                 | H45  | 61 60 53              |
| E214          | H46                                           | H46                               | H46  | 73 68 59              |
| E346          | H47                                           | H47                               | H47  | 99 83 99              |
| E247          | H48                                           | H48                               | H48  | 89 91 82              |
| E248          | H49                                           | H49                               | H49  | 87 87 87              |
| E372          | H51                                           | –                                 | H51  | 84 63 56              |
| E373          | H52                                           | –                                 | H52  | 85 67 59              |
| E374          | H53                                           | H53                               | H53  | 72 58 74              |
| E377          | H54                                           | –                                 | H54  | 81 68 70              |
| E375          | H55                                           | –                                 | H55  | 63 – –                |
| E375M*        | H55                                           | rough                            | H55  | 71 71 65              |
| E376          | H56                                           | –                                 | H56  | 65 40 53              |
| 09-1760*      | Untypeable                                    | H21                              | H21  | 97 95 96              |

*, serotyping titration or MS identification was not reached.

aSerotyping and MS-H were performed concurrently from subcultures of single bacterial colonies. MS-H was repeated on two consecutive days; bmotility induction was performed for these inconsistent strains after initial MS-H; cuntypeable by serotyping although previous MS and PCR-based sequencing showed type H21; dusing designated antisera by MS-H.

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However, the peptide sequence-based MS-H method does appear to show some marked improvements over antiserabased serotyping. Serotyping must withstand many stringent conditions relative to MS-H, such as motility induction which can be time-consuming, and the quality of serological reagents. For instance, antisera characteristics play an important role in serotyping, and ultimately affect the overall capacity of the assay. The MS-H method does not routinely require motility induction of *E. coli*, and uses far fewer reagents besides not using antisera, both of which make MS-H more straightforward to perform and less time-consuming to finish. In addition, based on the observations through EM and SDS-PAGE that flagella production by *E. coli* may vary and the quantity of extracted flagella may differ between strains. Although this heterogeneity of flagella production and dynamics of motility were considered major factors affecting serotyping, the MS-H method proved to be more tolerant to these changes, albeit with a lower detection limit and higher sensitivity.

MS-H can also be used for “sluggish” or inactive growing cultures, rough strains, and small volumes of culture as long as enough amounts of flagella can be extracted from the bacteria.

| Strain number | Previously recorded serotypes with motility induction | MS-H types without motility induction | Sequence coverage (%) |
|---------------|-----------------------------------------------------|--------------------------------------|-----------------------|
|               |                                                     |                                      | Run 1 | Run 2 | Run 3 |
| 09-1336       | NM                                                  | N/A                                  | N/A | N/A | N/A  |
| 09-1337       | NM                                                  | N/A                                  | N/A | N/A | N/A  |
| 09-1338       | NM                                                  | N/A                                  | N/A | N/A | N/A  |
| 09-1339       | NM                                                  | N/A                                  | N/A | N/A | N/A  |
| 09-1345       | NM                                                  | N/A                                  | N/A | N/A | N/A  |
| 09-1346       | NM                                                  | N/A                                  | N/A | N/A | N/A  |
| 09-0411b      | H11                                                 | H11                                  | 81  | 81  | 81   |
| 09-1342b      | H11                                                 | H11                                  | 83  | 82  | 83   |
| 09-1344b      | H11                                                 | H11                                  | 76  | 74  | 78   |
| 09-1765b      | H11                                                 | H11                                  | 98  | 98  | 91   |
| 09-1767b      | H11                                                 | H11                                  | 93  | 93  | 93   |
| 09-0409       | H28                                                 | H28                                  | 70  | 79  | 75   |
| 09-0410       | H28                                                 | H28                                  | 68  | 73  | 74   |
| 09-0416       | H28                                                 | H28                                  | 76  | 77  | 78   |
| 09-0416       | H28                                                 | H28                                  | 76  | 77  | 78   |
| 09-0412       | H49                                                 | H49                                  | 88  | 89  | 89   |
| 09-0413       | H16                                                 | H16                                  | 74  | 77  | 78   |
| 09-0415       | H8                                                  | H8                                   | 98  | 91  | 90   |
| 09-1347       | H19                                                 | H19                                  | 70  | 71  | 75   |
| 09-1348       | H19                                                 | H19                                  | 67  | 69  | 68   |
| 09-1353       | H25                                                 | H25                                  | 67  | 70  | 72   |
| 09-1354       | H25                                                 | H25                                  | 64  | 74  | 78   |
| 09-1770       | H19                                                 | H19                                  | 81  | 88  | 78   |
| 09-1774       | H19                                                 | H19                                  | 88  | 88  | 88   |
| 09-1764       | H19                                                 | H19                                  | 86  | 88  | 82   |
| 09-1766       | H34                                                 | H34                                  | 59  | 59  | 59   |
| 09-1768       | H14                                                 | H14                                  | 98  | 98  | 98   |
| 09-1769       | H14                                                 | H14                                  | 98  | 98  | 98   |
| 09-1775       | H25, then rough                                     | H25                                  | 76  | 81  | 80   |

NM, non-motile; N/A, not attainable.

*The same flagella digest was tested by LC-MS/MS three times within a one-week period; a two-year old residual digest was re-used.*

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### Table 6. Comparison of H serotyping and MS-H of *E. coli*.

| Parameter                        | H Serotyping                      | MS-H                                      |
|----------------------------------|-----------------------------------|-------------------------------------------|
| Diagnostic sensitivity           | Could reach 100%                   | Could reach 100%                          |
| Diagnostic specificity           | Could reach 100%                   | Could reach 100%                          |
| Analytical sensitivity           | Loop size of culture              | 5 colonies                                |
| Analytical specificity           | Antigenic epitope dependent        | Ionization dependent                      |
| Read-out                        | Agglutination titer observation by eyes; process may require several steps | Protein and peptide sequences analyzed by software; one step identification |
| Motility induction               | Routinely required                 | Not routinely required                     |
| Rapidity to get result           | 3 to 5 days                        | 4 hr for a single sample                   |
| Ease of identifying rough strains| Impossible                         | Possible; motility induction can be used to obtain result |
| Result consistency               | Motility induction and antisera dependent | Instrumentation and software dependent |
| Robustness and ruggedness        | Limited; largely performed manually; may require optimal antigen/antisera reaction conditions | Good; largely performed by machine; can tolerate wide range of sample amounts and different instruments |
| Throughput                       | Limited, largely performed manually; not easily repeated | Good; LC-MS/MS can run day and night; easy to repeat and obtain a better result |
| Sample stability                 | Variation in bacteria growth       | Protein digests are stable                 |
| Consumables and labor used       | Antisera, culture media, Craigie tubes; Half day of labor | Trypsin, lysozyme, nano-LC columns; instrument service contract; MS routine runs, half hour of labor |
| System suitability/accessibility | Reference labs/institutions with antiserum or antibody production | Institutions or service labs with MS capability |

In conclusion, advantages of the MS-H method described in this study are primarily high specificity, sensitivity, accuracy, and reproducibility. The approach is rapid, simple, and reliable. MS-H can be used independently to type *E. coli* flagella without motility induction. In addition, by avoiding the traditional methods of motility induction and multi-step agglutination reactions, results are generated much faster with greater simplicity than antibody-based agglutination and/or primer-based PCR. Lastly, the MS-H method should be particularly useful during *E. coli* outbreak situations to provide presumptive H type classifications.

### Materials and Methods

#### Bacterial strains and isolates

All the bacterial strains and isolates were from the ISO-certified national enteric reference center of National Microbiology Laboratory, Public Health Agency of Canada. The clinical isolates were originally from Alberta Provincial Laboratory for Public Health.

#### Flagella purification and on-filter digestion

*E. coli* bacteria were grown at 37°C overnight on TSA plates with 5% sheep blood. A full loopful culture on a 10 micro-liter loop was diluted in 1 ml of water containing 2 mg of lysozyme and gently suspended using a pipette tip. The suspension was incubated at room temperature for 10 min. Then the sample was vortexed at a maximum speed on a vortex mixer (Vortex-Genie 2, VWR) for 20 sec each time with 1 min break after vortexing for a total of 3 cycles of vortexing. After centrifugation for 20 min at 16,000g on a bench-top centrifuge (appendix 5417C), the supernatant was gently collected using a 1 ml syringe and passed through a 13 mm diameter filter with a 0.20 μm pore size (Acrodisc, PALL). The filter was washed with 3 ml of water and then flushed with air using a 1 ml syringe. 100 μl of trypsin (Promega mass spec grade, 100 μg per ml in 100 mM ammonium bicarbonate) was applied to the filter for digestion at 37°C for 2 hrs. The filter was flushed with 600 μl of water followed by air to collect the digest. For QSTAR MS detection, digests were dried down in a vacuum dryer and were reconstituted in 20 μl buffer A solution (0.1% formic acid used in nano-LC). For Orbitrap MS detection, 5 μl digests were directly mixed with 5 μl of 2x buffer A (0.2% formic acid) before loading.

Intact flagella were prepared by ultracentrifugation of the above supernatant at 50,000xg for 1 hr at 4°C [13] after lysozyme treatment and vortexing step shown above. The flagella pellet was then washed with 1 ml of cold PBS, spun down at 50,000g for 1 hr at 4°C, and finally dissolved in 100 μl of 100 mM ammonium bicarbonate for protein quantification with a BCA kit (Thermo-Fisher). Trypsin was added to the purified flagella at a 1:10 enzyme to protein ratio (in micrograms) for overnight digestion at 37°C and the digest was diluted with 2x buffer A for MS-H.

For side-by-side comparison of serotyping and MS-H, *E. coli* bacteria were grown from a single colony of culture from frozen stocks into two plates. One plate will be used for serotyping, and the other will be used for MS-H. For detection limit test of MS-H, cells from the single colony will be diluted in series with LB broth in triplicate, and the dilutions will be sub-cultured on TSA plates overnight at 37°C. The colonies will counted next day to convert to the cell numbers contained in the single colony used a day earlier. Certain numbers of colonies (5 to 500) will be picked for infilter flagella extraction and tryptic digestion.

### LC-MS/MS

For the QSTAR LC-MS/MS system, the 600 μl tryptic digest was vacuum-dried and 20 μl of buffer A was added to the digest. After 15 min equilibration with buffer A, 10 μl of the sample was loaded on to a 0.3×5 mm C18 pre-column (Agilent) for pre-binding and the pre-column was washed with buffer A for 5 min. The pre-column was then automatically switched to connect to a nano-LC-column. Nano-LC (Agilent) separation was run at 300 nL/min on a 0.075×15 mm C18 nano-column (Agilent) with a 55 min acetonitrile gradient from 5 to 35 percent, followed by a
10 min flush with 95% acetonitrile before equilibration with buffer A. MS data was collected from a triple-quadrupole-time-of-flight mass spectrometer (QSTAR-XL, ABSciex) with an information-dependent acquisition (IDA) method. A one-second parent ion scan followed by three 3-second product ion scans (i.e. a scanning cycle) were used to collect the tandem mass spectra of the 5 strongest ions from each scanning cycle [25]. For the Orbitrap system, 5 μl of the 600 μl flagellin digest was mixed with 5 μl 0.2% formic acid and then loaded on to a 0.1×2 mm C18 pre-column (ThermoFisher) for binding after a 15 min equilibration time with buffer A. The pre-column was washed for 5 min with buffer A and switched to connect to a nano-LC column (ThermoFisher). Nano-LC (Proxeon EASY-nano-LC, ThermoFisher) separation was run at 300 nl/min on a 0.075 mm 10 cm C18 nano-column with a 55 min acetonitrile gradient from 5 to 35 percent, followed by a 10 min flush with 95% acetonitrile before equilibration with buffer A. MS data was collected from an LTQ-Orbitrap XL system (ThermoFisher) with an IDA. One profile ion scan followed by 5 product ion scans (i.e. a scanning cycle) were used to collect the tandem mass spectra of the 5 strongest ions from each scanning cycle [19].

E. coli flagellin custom database creation and database search
A FASTA-formatted database for E. coli H types was created using the sequences and serotype information found in the NCBI protein database. Redundant sequences were collapsed into a single entry. The H type was listed in the sequence description. If no H type was specified in the NCBI database, the sequence was compared by BLASTp analysis against the sequences for which the H type was known, and the H type for the top blast result was used. In some cases the H-type was manually assigned (based on literature search) to sequences with missing H-type in NCBI, or assigned to sequences with incorrect H-type listed in the NCBI entry. Incorrect H-types were also discovered by finding outliers in a phylogenetic analysis of all E. coli flagellin sequences in the database. The final flagellin database had 196 protein sequence entries, and each entry contains a flagellin protein sequence of a specific H type (Fig. S1). The more common types, such as H7 and H11, have more entries (slightly different in amino acid composition due to some mutations) based on more studies on these types. Each entry has many theoretical trypptic peptides for protein identification and variable unique peptides to differentiate H types. This database was used to search the raw data in parallel with NCBIhr using Mascot (Matrix Science) search engine. The search parameters of 0.3 Dalton mass error tolerance for parent ions and 0.8 Dalton mass error tolerance for product ions were chosen for QSTAR data [25]. For Orbitrap data, 30 ppm mass error tolerance for parent ions and 0.5 Dalton mass error tolerance for product ions were chosen [19]. In all cases, two missed cleavages of trypsin digestion were used. Oxidation on methionine and deamidation on glutamine and asparagines were chosen as possible modifications. The top Mascot scoring hit was used to decide the H type. If more than one H type was present in the top scoring hits, the result would be considered ambiguous. The protein database and all peptide data are available in the supporting information (Protein Database S1 and Representative Peptide Data S1, respectively).

Electron microscopy
E. coli culture was gently mixed with fixative containing buffered 2% glutaraldehyde and 1% paraformaldehyde. The sample was then adsorbed to a glow discharged carbon-coated formvar film on a 400-mesh copper grid for 1 min, and negatively contrasted with 2% methylamine tungstate (Nano-W; Nanoprobes, Yaphank, NY, USA). Specimens were imaged in a FEI Tecnai 20 transmission electron microscope operating at 200 kV. Digital images of the specimens were acquired by an AMT Advantage XR 12 CCD camera (AMT, Danvers, MA, USA).

E. coli H Serotyping
The E. coli H antigen was serotyped based on the methods of several publications [22,26-28] summarized for our standard operation procedure. Basically, for motility induction, the bacteria were plated on MacConkey agar to check for purity and a single colony was selected. This colony was subcultured to a 0.25% Craige tube and incubated overnight at 35°C ± 2°C. Motile E. coli bacteria should travel through the Craige tube and up through the media using their flagella, while developing their H antigen. E. coli was then selected from the top of the media and transferred to a 0.3% Craige tube to further develop motility after incubation overnight at 35°C ± 2°C. To prepare the H antigen, Ewing’s broth was added to the top of the 0.3% Craige tube and gently drawn up and down so that the most motile bacteria originally at the surface of the Craige tube became suspended fully into the Ewing’s broth. The suspension was incubated at 35°C ± 2°C for approximately 4 hours and treated with formalin to kill the live bacteria and preserve the H antigen. The H antigen was diluted and screened first in antisera pools prepared with 5 to 8 individual monovalent antisera. For any pool with a positive reaction, individual monovalent antisera were tested. Absorbed antisera were used for final confirmation of the H serotype for any occasional strains that cross-reacted with more than one monovalent antisera. All antisera had been previously titred with reference E. coli strains. A positive H serotype was obtained when the H antigen had an agglutination equivalent to or better than the reference titre for that antisera. Serotyping synchronized with MS-H for comparison was done without motility induction, and was proceeded directly from a single colony subculture from frozen stocks with targeted antisera based on known H types through earlier serotyping and primary MS-H.

Sequencing of flic for H21
Oligonucleotides used for PCR based sequencing of flic for H21 are listed in Table S2. DNA amplification was performed using Platinum High Fidelity Taq (Invitrogen) kit as per manufacturer’s instructions. The reaction mix included deoxynucleotide triphosphates at a final concentration of 200 mM and the oligos JHF2 and JHR2 at a concentration of 200 mM for H21 DNA amplification, together with the reaction buffer supplied from the kit. PCR conditions were: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 68°C for 2 min, with a final extension at 68°C for 5 min. PCR products were purified using Montage PCR spin columns (Millipore) and sequenced on an ABI 3730 (Applied Biosystems) using PCR primers (JHF2 and JFR2) and sequence specific primers (H21F3 and H21R3). Sequence data were analyzed using DNAsStar Lasergene 7 (DNASTAR). The resulting consensus sequences were subjected to BLAST search to determine similarity to published sequences.

Supporting Information
Figure S1 Database of reference flagellin protein sequences and their known H antigen serogroups. The X-axis represents the number of unique protein sequences obtained. The Y-axis represents all 53 known H type serogroups. The final flagellin database contained 196 sequences.
Figure S2 Electron microscopy images of *E. coli* flagella. a. Reference *E. coli* strain E179 (H11) that lost flagella
growth after long-time storage. b. Reference *E. coli* strain E 170 (H2) flagella. c. Clinical *E. coli* non-motile isolate (09-1339) with
no flagella. d. Clinical *E. coli* motile isolate 09-1353 (H25) flagella. (DOCX)

Figure S3 SDS-PAGE of intact flagellin. Coomassie blue staining of a 4-12% gradient SDS-PAGE gel showing the variable
amounts of flagellin and purity of flagellin produced from a 10 μl loopful cell culture and extracted by ultracentrifugation from four
*E. coli* strains representing different H types. 10 μl of the 100 μl extracted protein were loaded onto the SDS-PAGE gel. Strains
used were: H7, 87-1215; H17: E185; H37: E205; H56: E376. A BCA kit was used to determine the total amount of flagellin
extracted from each strain, which is labeled on the X-axis under each H type. (DOCX)

Table S1 Analytical sensitivity test for MS-H of purified flagellin tryptic digests on QSTAR platform. Reference strain 87-1215 (O157:H7) was cultured overnight at 37°C and intact flagella were purified by ultracentrifugation as shown in Materials and Methods. The flagella were dissolved in 100 μl of 100 mM ammonium bicarbonate for protein quantitation with a BCA kit. Trypsin was added at a 1:10 enzyme to protein ratio for overnight digestion at 37°C. The digest was diluted with 2x buffer A and designated amounts of the protein digest were loaded onto the LC-MS/MS system for MS-H. (DOCX)

Table S2 Primers used for H21 sequencing. (DOCX)

Table S3 Real-time comparison of H serotyping and MS-H of flagella extracted from four selected *E. coli* strains. Flagella were extracted from *E. coli* for LC-MS/MS in parallel with motility induction and serotyping independently on day 2, 7 and 16. MS-H was performed on the QSTAR system. (DOCX)

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Author Contributions

Performed electronic microscopy: DB TB. Aided database creation: SM GVD. Conceived and designed the experiments: KC G. Wang. Performed the experiments: JM LP DL RN AG DJ. Analyzed the data: KC G. Wang. Wrote the paper: AS LG HT G. Westmacott MG MD KC G. Wang.
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