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# Analysis of Whole-Cell Fatty Acid Profiles of Verotoxigenic *Escherichia coli* and *Salmonella enteritidis* with the Microbial Identification System

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**Differentiation of strains within bacterial species, based on gas chromatographic analysis of whole-cell fatty acid profiles, was assessed with 115 strains of verotoxigenic *Escherichia coli* and 315 strains of *Salmonella enteritidis*. Fatty acid-based subgroups within each of the two species were generated. Variability of fatty acid profiles observed in repeat preparations from the same strain approached that observed between subgroups, limiting the usefulness of using fatty acid profiles to subgroup verotoxigenic *E. coli* and *S. enteritidis* strains.**

Illnesses caused by food-borne microorganisms are serious health problems throughout the world. Verotoxigenic *Escherichia coli* (VTEC) and *Salmonella enteritidis* are two examples of food-borne pathogens capable of causing disease.

Rapid test methods that can accurately differentiate strains of food-borne pathogens could be used to track the distribution of strains that are responsible for disease outbreaks and help in the study and control of food-borne diseases. Multiple methods have been used to characterize bacterial food-borne pathogens. These include serotype, phage type, biotype, toxin type, plasmid profile, antibiotic resistance profile, multilocus enzyme electrophoresis, lipopolysaccharide profile, outer membrane protein profile, rapid amplification of polymorphic DNA, restriction endonuclease digestion, and ribotype methods (1, 2, 5, 7, 9, 13, 14, 16, 17). Many of these techniques are labor-intensive and/or require specialized skills, which makes them less suitable for routine laboratory analysis. In addition, some bacterial isolates are untypeable by methods such as phage typing.

The comparison of fatty acid profiles from whole bacterial cells is an approach worthy of investigation for its potential ability to inexpensively and rapidly differentiate bacterial strains and its ability to analyze a wide spectrum of microorganisms. The Microbial Identification System (MIS; Microbial ID Inc. [MIDI], Newark, Del.) uses gas chromatography to analyze whole-cell fatty acid profiles. The MIS was designed for microbial species identification, but it has been suggested that the system may also be capable of epidemiological typing of strains (3, 12). Mukwaya and Welch (12) used the MIS to track the sources of *Pseudomonas cepacia* strains isolated from cystic fibrosis treatment centers, and Birnbaum et al. (3) used the MIS to estimate the relatedness of a large number of coagulase-negative staphylococcal strains. Using the MIS as a typing system would be even more advantageous if fatty acid profiles could be demonstrated to be associated with virulence characteristics.

The objectives of the present study were as follows: (i) to develop fatty acid-based typing libraries for *S. enteritidis* and VTEC based on single fatty acid analyses of representative isolates of each species; (ii) to assess the repeatability of fatty acid profile results of *S. enteritidis* and VTEC when strains are grown under standardized conditions, and (iii) to identify associations between fatty acid groupings of bacteria and the history or characteristics of the isolates.

A total of 115 strains of VTEC and 315 strains of *S. enteritidis* from the culture collection of the Health of Animals Laboratory, Health Canada, were included in this study. Most of the VTEC and *S. enteritidis* strains were collected during surveillance programs of Ontario dairy and beef cattle and Canadian poultry farms, respectively. The serotypes, toxin types, and presence of attaching and effacing genes for VTEC strains and the phage types, plasmid profiles, and biotypes of the *S. enteritidis* strains were determined at the Health of Animals Laboratory (14). All strains were subcultured at least twice on Trypticase soy broth agar (TSBA; BBL, Cockeysville, Md.) plates at 28°C and then stored on TSBA slants at 4°C until they were used in the experiments.

The strains were grown on quadrant-streaked TSBA plates for 24 ± 1 h at 28 ± 1°C, the growth conditions recommended for the MIS's TSBA library (11). The cells (45 ± 1 mg [wet weight]) were harvested from the third quadrant of the TSBA plates. If the cells were not processed within 30 min of harvesting, they were stored at −80°C until fatty acid extraction and analysis. The harvested bacterial cells were treated chemically to extract and convert the fatty acids present in the cell wall or cell membrane fractions of the bacteria to fatty acid methyl esters (FAMEs). Sample preparation consisted of four steps, namely, saponification, methylation, extraction, and an alkaline wash, as described by MIDI (11). The FAMEs were injected into a Hewlett-Packard 5890 series II gas-liquid chromatography column equipped with a flame ionization detector, which acted to separate and quantify the different FAMEs in the mixture. Calibration standards, a negative control blank, and a positive control culture preparation were run with each batch of samples, as recommended by MIDI (11). The retention times of the sample's FAMEs, relative to those of the calibration standards, were used by the MIS computer pro-

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TABLE 1. Summary of repeatability of fatty acid profiles determined by repeat analysis of one type strain each of VTEC and *S. enteritidis*

| Data point and strain | ED | | | | |
|---|---|---|---|---|---|
| | Mean (U) | SD[a] (U) | CV[b] (%) | Range (U) | 95th %ile (U) |
| Readings of split samples | | | | | |
| VTEC | 0.46 | 0.29 | 63 | 0.13–1.35 | 1.01 |
| *S. enteritidis* | 0.34 | 0.18 | 53 | 0.09–0.77 | 0.76 |
| Replicate plates incubated in parallel | | | | | |
| VTEC | 2.31 | 1.66 | 72 | 0.20–6.87 | 6.11 |
| *S. enteritidis* | 2.04 | 1.29 | 63 | 0.46–4.62 | 4.49 |
| Two batches of medium | | | | | |
| VTEC | 2.68 | 1.61 | 60 | 0.38–5.99 | 5.81 |
| *S. enteritidis* | 2.28 | 1.02 | 45 | 0.84–4.50 | 4.46 |
| Weeks, common subculture | | | | | |
| VTEC | 4.38 | 2.20 | 50 | 0.44–8.81 | 8.71[c] |
| *S. enteritidis* | 2.13 | 1.59 | 75 | 0.45–6.64 | 5.97[c] |
| Sequential subcultures | | | | | |
| VTEC | 4.30 | 2.47 | 57 | 0.91–8.95 | 8.62 |
| *S. enteritidis* | 2.50 | 1.20 | 48 | 0.60–4.80 | 4.68 |

[a] SD, standard deviation.
[b] CV, coefficient of variation.
[c] Cutoff ED values used for library generation in the respective species (8.7 for VTEC and 6.0 for *S. enteritidis*).

gram to identify the different fatty acids in the sample preparation.

An electronic database of one fatty acid profile per isolate was established for each species studied, with the MIS library generation computer software. The MIS software included a dendrogram program which used cluster analysis to produce unweighted pair matchings of samples based on the named fatty acid composition of samples. These dendrograms graphically displayed the similarity of fatty acid profiles from different sample preparations in the form of a tree structure. Similarity between different fatty acid profiles was measured in euclidian distances (EDs), a statistical measure of similarity (11). Species-specific dendrograms were prepared from the *S. enteritidis* and VTEC single fatty acid analysis databases. These dendrograms showed subgroups based on differences in fatty acid profiles which occurred within each species and the ED at which these subgroups occurred. To determine the ED cutoff at which reproducible subgroups could be generated from these dendrograms, it was necessary to determine the level of variability inherent in the analysis of *S. enteritidis* and VTEC fatty acids.

The repeatability (precision) of fatty acid profile analyses was analyzed by repeated testing of one type isolate each from VTEC and *S. enteritidis*. The type isolates were selected from the center of dendrograms generated from the fatty acid profiles of all strains examined within each species, by using the rationale that strains in this center position would be the most representative in their fatty acid profiles. The two type isolates were then subjected to repeated testing over a 3-week period in the following manner. Two separate batches of medium plates were prepared independently and stored at 4°C for use in parallel through out the 3-week study. During each week, each isolate was subcultured daily onto fresh TSBA plates, beginning with a 24-h culture plated on Trypticase soy agar with 5% sheep blood (BBL). The second, fourth, and sixth subcultures were assessed to represent the sequential passage of a strain under laboratory conditions, as might be encountered in an epidemiological trace back investigation. Each of the subcultures tested was streaked onto two plates of each of the two different batches of media. Following growth and harvesting of the cells and FAME preparation, each sample preparation was split into two vials and the fatty acid profiles were assessed separately by the MIS. This led to the generation of 72 data points for each strain during the 3-week experiment (3 weeks in the experiment, three subcultures per week, two batches of medium per subculture, two plates per batch of medium, and two gas chromatographic separations per sample preparation). Dendrograms were generated for the type strains of each species encompassing unweighted paired matchings of fatty acid compositions among repeat readings of (i) the same sample preparation, (ii) identical isolate and subculture and medium batch pairs incubated side by side in the same incubator on two different plates, (iii) identical isolate and subculture and medium batch pairs incubated on different days (1 week apart), (iv) identical isolate and subculture pairs incubated on different batches of the same medium, and (v) different subcultures of the same isolate on the same batch of medium. Table 1 summarizes the means, standard deviations, coefficients of variation, ranges, and the 95th percentiles of the distributions of EDs within these data points.

The physical repeatability of the MIS or Hewlett-Packard gas chromatography measurement system was observed to be excellent, demonstrating an overall mean ED between split-sample preparations of only 0.46 ED units, ranging from 0.09 to 1.35 ED units for both type strains. The biological variability between repeated runs of individual isolates, however, was high for both species type isolates. It ranged from 0.2 to 8.95 ED units in repeated testing of identical isolates and was not controlled sufficiently through the use of standard protocols.

Critical ED cutoffs were selected for VTEC and *S. enteritidis* strains based on 95th percentiles of the variability observed in repeat measurements of the type strains (Table 1) and used to create *S. enteritidis* and VTEC specific fatty acid-based libraries containing different subgroups. A cutoff value of 8.7 ED units was used to distribute the 115 isolates of VTEC into three different subgroups. This grouping is depicted schematically in a dendrogram (Fig. 1). A cutoff value of 6.0 ED units was used to divide the 315 isolates of *S. enteritidis* into six different

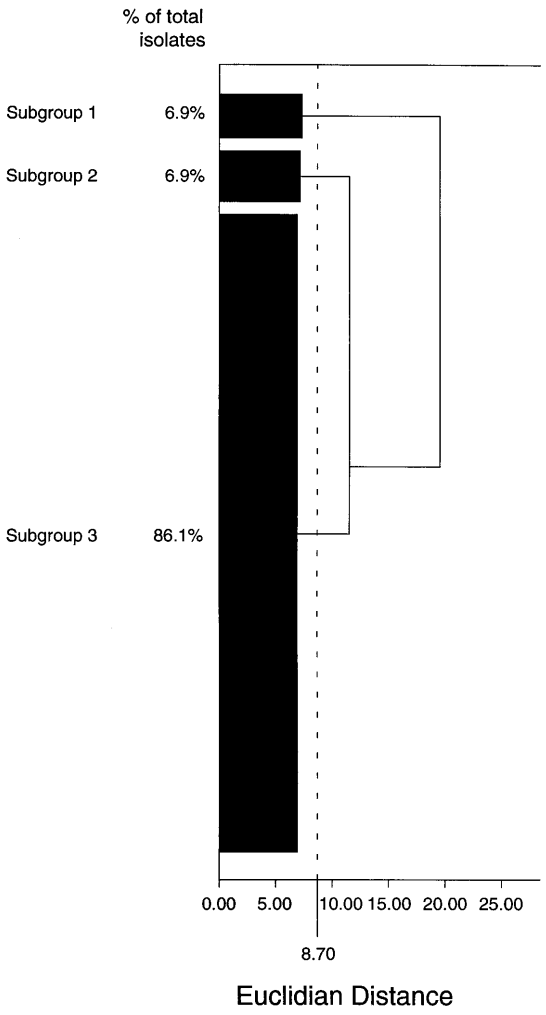


FIG. 1. Schematic representation of MIS dendrogram of VTEC isolates.

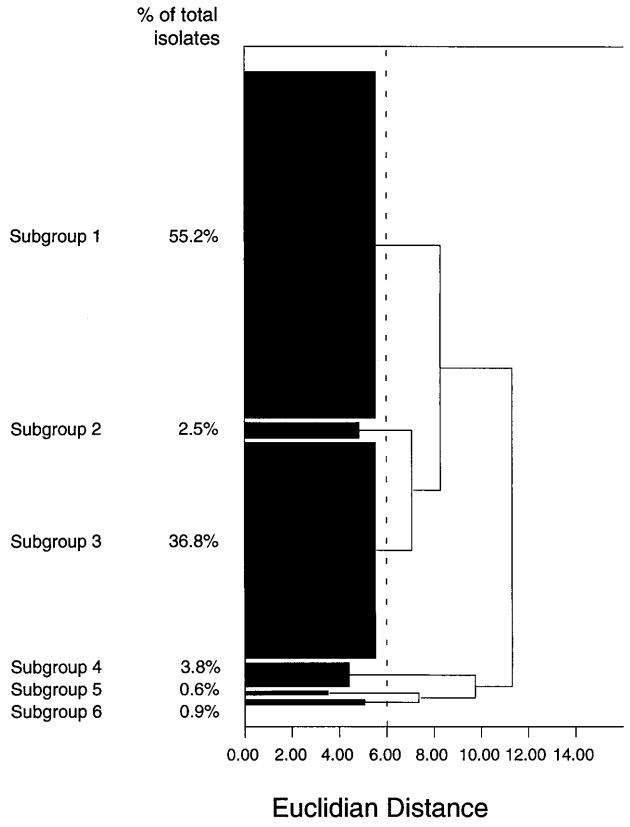


FIG. 2. Schematic representation of MIS dendrogram of *S. enteritidis* isolates.

subgroups. This grouping is depicted schematically in a dendrogram (Fig. 2). The high variability level of fatty acid profiles was reflected in the library subgroupings, where the majority of strains were included within a small number of subgroups for both species, limiting the discriminatory capacity of the system when used as a typing tool for these species. One VTEC subgroup contained 86.1% of the isolates tested, and both of the remaining subgroups contained eight isolates each. *S. enteritidis* subgroup 1 contained 55.2% of the isolates, and subgroup 3 contained 36.8% of the isolates. Each of the remaining subgroups contained 12 or fewer isolates.

Chi-square and Fisher's exact tests were used to assess the statistical significance of potential associations observed between isolate fatty acid-based subgroupings and the history or characteristics of each isolate, including source of isolate, serotype, toxin type, presence of the attaching and effacing genes, and the form of isolation for VTEC isolates and biotype, phage type, and plasmid profile (plasmid groups and individual plasmids) for *S. enteritidis* isolates. None of the statistical tests was found to be significant at the 5% level among characteristics that included at least four isolates.

In any system of measurement, it is important to understand the precision of the system to avoid erroneous declarations of similarity or difference between measurements. The overall variability observed in the results of any test system is caused by a combination of variability in the measurement system and by the true variability of the substance being measured. The phenotypic expression of fatty acids within bacterial cell walls or cell membranes is influenced by a number of factors, including medium composition, growth temperature, and rate of growth (4, 6, 8, 10); thus, protocols must be standardized before fatty acid profiles can be compared. Even under standardized conditions, the variability (or limits to the precision) must be understood. Steele et al. (15) investigated the repeatability of the MIS with five *Listeria monocytogenes* strains which were representative of the spectrum of fatty acid profiles observed within that species. The level of variability of fatty acid profiles generated from the same bacterial strain was found to approach the level of variability observed between different strains, thus limiting the usefulness of this system for studying the epidemiology of *L. monocytogenes* isolates. These results are in contrast to those of a study which used the MIS to examine the relatedness of 200 isolates of coagulase-negative staphylococci (3). These authors found the system to be a useful screening tool that could be used before the use of more time-consuming and expensive typing methods.

The usefulness of the MIS as a typing tool may be dependent upon the species examined. Some species may exhibit more-reproducible and more-varied fatty acid profiles than others. The results of this study suggest that at present, single assessments of fatty acid profiles for the purposes of VTEC or *S. enteritidis* strain differentiation should be interpreted with caution. The variability reported here appears to be due to the biological variability in phenotypic expression of fatty acids by

individual isolates, even when cultivated under standardized, controlled conditions. This limits the usefulness of reference libraries of fatty acid profiles that have been developed with single assessments of each isolate to distinguish between strains within each of these species. Developers may wish to refine computer algorithms in such systems to allow the analysis of repeated measurements of given isolates. The calculation of means and confidence intervals describing the fatty acid profiles (in as many as 20 dimensions), observed in repeated measurements, might better facilitate the application of fatty acid analysis in the differentiation of strains of food-borne pathogens.

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