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Metabolic parameters linked by phenotype microarray to acid resistance profiles of poultry-associated *Salmonella enterica*

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Abstract

Phenotype microarrays were analyzed for 51 datasets derived from *Salmonella enterica*. The top 4 serotypes associated with poultry products and one associated with turkey, respectively Typhimurium, Enteritidis, Heidelberg, Infantis and Senftenberg, were represented. Datasets were partitioned initially into two clusters based on ranking by values at pH 4.5 (PM10 A03). Negative control wells were used to establish 90 respiratory units as the point differentiating acid resistance from sensitive strains. Thus, 24 isolates that appeared most acid-resistant were compared initially to 27 that appeared most acid-sensitive (24 × 27 format). Paired cluster analysis was also done and it included the 7 most acid-resistant and -sensitive datasets (7 × 7 format). Statistical analyses of ranked data were then calculated in order of standard deviation, probability value by the Student's *t*-test and a measure of the magnitude of difference called effect size. Data were reported as significant if, by order of filtering, the following parameters were calculated: i) a standard deviation of 24 respiratory units or greater from all datasets for each chemical, ii) a probability value of less than or equal to 0.03 between clusters and iii) an effect size of at least 0.50 or greater between clusters. Results suggest that between 7.89% and 23.16% of 950 chemicals differentiated acid-resistant isolates from sensitive ones, depending on the format applied. Differences were more evident at the extremes of phenotype using the subset of data in the paired 7 × 7 format. Results thus provide a strategy for selecting compounds for additional research, which may impede the emergence of acid-resistant *Salmonella enterica* in food.

Keywords: Salmonella; Food safety; Phenotype; Acid tolerance; Metabolism; Poultry

1. Introduction

In the US, 30 serovars of *Salmonella enterica* causing foodborne illness persist at an incidence in the general food supply to warrant continuous survey [1]. Several serovars are of interest for their association with eggs and poultry products. Phenotype microarray (PM) data were accumulated for the top 4 serovars of *Salmonella enterica* linked to chicken (Typhimurium, Enteritidis, Heidelberg and Infantis) and one associated with turkey (Senftenberg) [1,4]. During accessioning of PM datasets, we observed that respiratory activity was present or absent in PM10 A03 (pH 4.5). The disparities between strains appeared large in comparison to other wells in the pH gradient (PM10 A01 − PM10 A12). The acid tolerance response is generally accepted as a fundamental characteristic

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of the Salmonellae that facilitates survival of the pathogen in a range of acidic environments, both internal and external to avian and mammalian hosts [9]. The acid tolerance response is complex in its induction, has variable determinants and has been associated with multiple inducers, genes and regulatory pathways [3,14,20,21]. Within the food industry, survival of *Salmonella* in acidic conditions is of great concern because low pH is used as a preservative [10,15,18]. We thus wanted to know if isolates that varied in acid tolerance had other linked metabolic differences. Also of value was the development of a general statistical strategy for processing large amounts of information derived from PM analysis of *Salmonella enterica* wildtype isolates.

2. Materials and methods

2.1. Description of bacteria

Bacteria used for this study were obtained from a variety of proprietary sources and chosen for inclusion based on a known association with poultry over a period of 3 years. *S. enterica* serotypes represented are Typhimurium (ST), Enteritidis (SE), Heidelberg (SH), Infantis (SI) and Seftenberg (SS). The first 4 serotypes have a strong association of foodborne illness and chicken products, whereas the last one is more associated with turkey [1]. Each serovar had multiple independent isolates, noted by a number following the serotype designation. Thus, SE7 was the 7th independent isolate of serovar Enteritidis assayed. Some isolates were repeatedly assayed to determine variation occurring between experiments over time. For example, SE7 was used as a primary control strain and it was assayed 10 times, and thus datasets were numbered SE7-1 through SE7-10 in tables. Other isolates that were assayed more than once include SE5 (4 runs), SI4 (2 runs), SI6 (2 runs) and SH11 (2 runs). Isolates were stored at −80 °C in 15% glycerol pending analysis.

2.2. Parameters of analysis

Runs were conducted in Omnilog SN248 (Biolog, Inc., Hayward, CA, USA) at 42 °C, which is the normal body temperature of poultry. Data were collected over 48 h. Isolates were revived from frozen stock onto Biolog universal growth (BUG) agar prepared with 5% sheep blood according to the provider's directions (Biolog, Inc.). Plates were incubated for 16 h at 37 °C and then placed at 4 °C for at least 1 h. Each isolate was processed individually in order to keep the temperature of media near 4 °C, which aids in keeping background respiratory activity minimized. Cells were suspended in IF-0 without dye (Biolog, Inc.) to a 42% suspension and then transferred according to PM directions to IF-0 with dye (PM1 through 8) or IF-10 with dye (PM 9 and 10). Suspensions were returned to 4 °C. One suspension at a time was plated to PM1-10, sealed with plastic and then kept at 4 °C until information had been entered and the machine was ready to load and run. Respiratory activity results in an irreversible dye reaction that is digitally captured every 15 min and converted to respiratory units (RU) by proprietary software (Biolog, Inc.). Average height was used for analysis of datasets.

2.3. Statistical analyses

2.3.1. Determination of background respiratory units (RU)

The A01 negative control wells from plates PM1, PM2A, PM3B, PM6, PM7 and PM8 were used to calculate average background RU from an initial 57 datasets (Table 1A). Any individual dataset that had an average for its 6 negative control wells exceeding background RU was excluded from analysis. Background RU is very sensitive to technical aspects of loading and handling plates and methods used to inhibit background RU as described above must be followed to maintain quality of any one run.

2.3.2. Initial cluster analysis and calculations for assessing significance

To begin analysis, data were first partitioned into two clusters based on results from 51 qualifying datasets as ranked from greatest to least by RU at PM10 A03, pH 4.5. This initial cluster is referred to as the 24 × 27 format, because 24 strains appeared acid-resistant and 27 strains appeared sensitive (Table 1B).

Three types of calculations were performed on the 24 × 27 formatted clusters, and these were filtered in order to observe the most likely differences in phenotype (Table 2). First, standard deviation (stdev) was calculated using the Excel STDEV.S function for each of the 950 chemicals. Chemicals that did not have at least one stdev of at least 24 RU across all datapoints were essentially homogeneous and of no significance. Then, the probability value (p) by Student's t-Test was calculated using Excel. Any chemical that did not have a p value less than 0.030 was excluded, or filtered out, as being significantly different. For the 24 × 27 format, tails = 1, type = 2 and for the paired 7 × 7 format, tails = 1, type = 1.

Effect size (ES) was also determined by calculating the average of values for two clusters and then dividing the difference of the averages by the standard deviation [5]. ES conveys the magnitude of difference between clusters, and the greater the ES, the greater the magnitude of the difference. ES assesses the magnitude of an effect based on absolute values, and guidelines suggest that minor effects are in the range of ±0.2 to 0.4, moderate effects are in the range of ±0.4 to 0.8, and major effects exceed ±0.8 [5,6]. For these analyses, a positive ES was associated with acid resistance and a negative ES was associated with acid sensitivity, because the formula used for each chemical was AR (average of the acid-resistant cluster) − AS (average of the acid-sensitive cluster) divided by the stdev calculated from AR and AS values combined. Only absolute ES values of ≤−0.50 or ≥0.50 were considered significant.

2.3.3. Additional cluster analysis for minimizing false-negatives

A second clustering strategy was used and it also filtered datasets in order of stdev, p value and ES as done for the