First step in using molecular data for microbial food safety risk assessment; hazard identification of Escherichia coli O157:H7 by coupling genomic data with in vitro adherence to human epithelial cells

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First step in using molecular data for microbial food safety risk assessment; hazard identification of *Escherichia coli* O157:H7 by coupling genomic data with in vitro adherence to human epithelial cells


1. Introduction

Microbiological risk assessment is part of an established framework for risk analysis that consists of the following steps: statement of purpose, hazard identification, hazard characterization, exposure assessment and risk characterization (CAC, 1999). In the field of food safety, a ‘farm to fork’ quantitative risk assessment (QMRA, Quantitative Microbial Risk Assessment) approach is often applied to assess the public health risk for a particular pathogen/ matrix combination (e.g. Romero-Barrios et al., 2013). Transmission of the pathogen (e.g. *Campylobacter* spp.) through a specific food production chain (e.g. poultry) may be quantified using a probabilistic QMRA model (e.g. Nauta et al., 2005). Variability and/or uncertainty in the pathogen prevalence, concentrations and food production process properties are included as model parameters. Monte Carlo simulations, or other probabilistic techniques, are used to predict public health risk and the effect of different intervention strategies can then be calculated to support industrial or governmental decision making (e.g. Pielaat et al., 2014). Systematic sensitivity analyses can be used to indicate the value of new evidence but only at the level of detail that was used during the model construction.

Since the introduction of high-throughput DNA sequencing technologies, however, food microbiology has moved beyond the assessment of microbial behavior in different food processes for agents classified at (sub)species and serovar level. Moreover, with the rapidly dropping costs of sequencing, whole genome sequencing (WGS) will soon
become a standard surveillance technique for the subtyping of isolates for epidemiological purposes. Although the use of molecular data has proved to be a powerful tool in decision making during outbreak investigations (Dallman et al., 2014; Underwood et al., 2013), the application of this data in microbiological risk assessment is currently an unexplored area in the public health domain. In recent years, a number of reviews and opinions have been published exploring the potentials of ‘omics techniques’ for MRA (Abee et al., 2004; Brul et al., 2012; Carriço et al., 2013; Havelaar et al., 2010; Pielaat et al., 2013a,b) but, evidence based research, as a first step to convert these heuristic approaches into normative tools for practical use, is still needed.

The difficulties associated with using molecular data for food safety risk assessment are complex but are related to the prescribed framework and the current methodology which generally expresses a large (but closed) joint probability to represent a ‘farm to fork’ hazard domain. For example, where the variability and/or uncertainty of concentration and prevalence data are relevant in QMRA these can be described by probability distributions but it is not clear how to use this approach when the data consists of a genome sequence. Firstly, new technologies provide information at a completely different level of description (genes or their products) that makes their joint probability, in its simplest form, unmanageable. Secondly, the new description does not, in the first instance, provide a clear connection between the observed quantities and the output measures, such as survival or health impacts, that are the object of risk assessments. So, for decision support, the biggest challenge facing genomics is the prediction of phenotypic properties of a particular pathogen within a food chain based on genotypic data. An understanding of systems biology is needed, as the organizational principle in pathophysiology, to describe the relation between the new level of genetic sequence data and the health endpoints of concern. Whereas in the established framework for risk assessment, the elements of a joint probability are considered to be known, or knowable, the introduction of a new level of description and a systems property leads to elements of a joint probability that cannot easily be formulated and to dependencies that are not easy to identify. To reduce the numbers of possible relations and translate genetic sequence into phenotypic properties, an understanding of pathogen physiology is needed. Currently, such understanding is incomplete and consequently the mapping of genetic sequences onto a quantitative description of risk is problematic: the number of genes outweighs the number of strain samples by many orders of magnitude. It should be clear that statistical analysis of WGS data is non-trivial, and that reproducible and meaningful associations between gene variability and phenotypic properties need to be established before genetic data can be used for decision making in food safety.

As indicated during EFSA’s 20th scientific colloquium (EFSA, 2014), a diversity of exemplary data analyses need to be developed and shared within the scientific community to allow for the identification and appreciation of “best practices” in moving forward from current methodology. A (theoretical) methodology for hazard identification is proposed that uses WGS data analysis to link genomic sequences with phenotypic behavior for Shiga toxin-producing Escherichia coli O157 (STEC O157) as a case study. This is achieved by the integration of genomic (single nucleotide polymorphism (SNP) genotypes) data with phenotypic (attachment to epithelial cells) information and with epidemiological data (outbreak strains and the epidemiological relationship with sporadic cases) in a Genome Wide Association Study (GWAS).

The aim of this study is to introduce a method for hazard identification that links WGS data with results on in vitro adherence to epithelial cells as a proxy for virulence using a subset of STEC O157 isolates as a case study. An explanation of the concepts, identification of the value of the methodology and a relationship with the public health domain are supported by a thorough discussion of further research needs. This paper identifies a necessary paradigm change in public health microbiological risk assessments.

2. Materials and methods

2.1. STEC O157 as a case study

STEC is of public health concern because of its ability to cause outbreaks and severe disease such as hemorrhagic colitis (HC) or hemolytic–uremic syndrome (HUS). Currently, different STEC serogroups are placed in different risk classes (i.e., seropathotypes) based on their epidemiological association with severe disease and outbreaks (Karmali et al., 2003). However, this system is of limited use for two reasons. First, it is retrospective, only including known types. Second, the pathogenicity of STEC cannot be predicted from the serotype alone. Numerous (putative) virulence genes have been associated with increased disease severity and individual strains of STEC can differ considerably in their virulence profile and, consequently, in their pathogenic potential (Delannoy et al., 2013). Gene association studies are normally conducted by linking the genetic content of the strain to the seropathotype or more specific to the clinical symptoms it caused (Andersson et al., 2011; Persson et al., 2007). However, these association studies might be confounded by food and host effects. Within serogroup O157 considerable attention has been given to the non-random distribution of genotypes among bovine and human clinical isolates, showing considerable genome divergence (Franz et al., 2014). However, observed non-random distribution of clades and lineages among bovine and human clinical isolates might be the result of a differentiation in virulence, transmission capacity and survival, or some combination (Franz et al., 2012). For a better understanding of STEC O157 risks, these (or other) potential causes should be investigated separately. Recently it was shown that the environmental exposure route selects for strains characterized by the absence of mutations in the general stress response system rpoS, which are subsequently more likely to survive the human gastric barrier (Franz et al., 2011; van Hoek et al., 2012).

The evaluation of intrinsic differences in virulence requires a standardized model system. Several animal models for STEC disease exist and their value is clearly recognized (Melton-Celsa and O’Brien, 2003). However, for technical, economic, and ethical reasons, in vitro models offer a relevant alternative to in vivo studies. Although more distinct from a human system, in vitro models offer more stability in terms of reproducibility (Berk, 2008). The combination with WGS information subsequently allows for genotype–phenotype matching and comparative genomics of strains in order to identify genetic elements that differentiate highly virulent strains from less virulent ones. Analysis at the SNP level is a straightforward approach to extracting elementary information on genotype, and will be used in this study.

2.2. E. coli O157 strains

In an earlier study the frequency of E. coli O157 genotypes among 73 bovine, 29 food, and 85 human clinical isolates was determined in The Netherlands (Franz et al., 2012). The results demonstrated that O157 lineages (as defined by the lineage specific polymorphism, or LSPA, assay) were non-randomly distributed among isolates of bovine and clinical human origin. A selection of in total 38 human and animals strains from different LSPA lineages was selected for further investigation in this study (Table 1).

2.3. Genotypic data

Whole genome sequences of the 38 E. coli O157 were obtained using the Illumina MiSeq platform with 2 × 150 (human isolates) and 2 × 250 (animal isolates) paired end runs.

The genomic sequence of a human Shiga toxin-producing E. coli O157:H7 strain isolated during the Sakai outbreak which occurred in Japan during 1996 was used as a reference. The short sequencing reads were mapped onto the reference chromosome (accession
number: NC_002695) and its large plasmid pO157 (accession number: NC_002128) using the alignment tool BWA (Li and Durbin, 2010). The SAMtools software package converted the SAM format files to BAM format and sorted the BAM files (Li et al., 2009). SAMtools mpileup generated BCF format files and bcftools was used to call the SNPs (between reference and samples) in VCF format. SNPs were filtered with the quality threshold set at a minimum read depth of 10. SNP data were transformed to binary, 0/1, data. Those sites where a SNP was identified in the STEC strains under study (test strains) compared to Sakai (reference strain) received a 1. A 0 was placed accordingly for identical nucleotides on the genome of each test strain compared to the reference strain Sakai. This resulted in a binary \( m \) by \( n \) matrix, where \( m \) is the number of SNPs and \( n \) the number of test strains (\( n = 38 \)).

2.4. Phenotypic data

The adhesive properties to human intestinal cells were used as a proxy for virulence in this study. In total, 18 human O157 isolates (H-numbers) and 20 animal O157 isolates (A-numbers) from lineage I, I/II and II (see Table 1 for strain properties) were investigated and compared. Differentiated Caco-2 cells were used as a representative model system for human intestinal cells. They were produced, treated and seeded as previously described by Oliveira et al. (2011). For adhesion experiments the cells were seeded in 12-well plates at a concentration of \( 1.6 \times 10^5 \) cells/well. Each STEC strain was inoculated in BHI broth and incubated overnight (ON) at 37 °C to obtain a culture consisting of approximately \( 10^6 \) CFU/ml. Subsequently, three decimal dilutions resulting in a STEC suspension of approximately \( 10^5 \) CFU/ml were made. From these last suspensions, Caco-2 cells in 12-well plates were inoculated with 40 \( \mu \)l per well, per STEC strain six wells. Plates were centrifuged (1 min at 175 \( \times \)g) and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2 for 1 h. 

After incubation and three washings with pre-warmed sterile phosphate-buffered saline (PBS), 1 ml 1% Triton-X100 in PBS (pre-warmed) was added to each well to detach the cells.

The detached cells were collected and the contents of three wells were combined. Decimal dilutions were prepared in peptone physiological salt solution, and the dilutions were plated on Brilliance™ E. coli/ coliforms Selective Agar (Oxoid, Badhoevedorp, The Netherlands).

3. Statistical data analysis

3.1. Phenotypic data

The fractional adherence was calculated by dividing the number of STECs after the adhesion assay by the number of STECs added to the Caco-2 cells. If cells are assumed to be homogeneously distributed in the ON culture, then the number of cell counts per dilution is Poisson distributed with a parameter \( \lambda \). Based on this assumption, the best estimate for \( \lambda \), the expected number of cells in a sample, can be estimated from counts in serial dilutions of the original sample according to

\[
\lambda = \frac{\sum_{i=1}^{k} x_i}{n}
\]

where \( n \) is the number of counts in the ith dilution. The point estimate for the fraction of bacteria attached to the Caco-2 cells is \( \frac{\lambda}{\lambda_1} \) where \( \lambda_1 \) is the expected number of bacteria in the overnight culture and \( \lambda_2 \) is the expected number of bacteria attached to the Caco-2 cells.

3.2. Simple linear regression

The basic idea in this study is to identify SNPs in the test strains that could be associated with an increased virulence behavior (represented by relatively high Caco-2 cell attachment fractions compared to other test strains without these SNPs).

The strength of this association can, in the most basic form, be estimated using the following simple linear regression model for each SNP:

\[
y_i = \mu + \beta x_i + \epsilon_i,
\]

whereby, \( y_i \) is the fractional Caco-2 adhesion for strain \( i \), \( \mu \) is the mean response, \( \beta \) is the SNP effect, \( x_i \) is an indicator variable with \( x_i = 0 \) if the marker (here, SNP) score of test strain \( i \) is equal to the reference strain and the residual errors, \( \epsilon_i \), have independent normal distributions with variance \( \sigma^2 \).

For each SNP the null hypothesis \( H_0: \beta = 0 \) is tested against an alternative hypothesis \( H_1: \beta \neq 0 \) and the P-values and effects \( \beta \) are calculated and transformed to a \(-\log_{10}\) scale.

In Genome Wide Association Studies (GWAS) a corresponding test is applied for all markers. In terms of hazard identification this simplified model would assign strains to one of two classes of hazards, with different rates of adhesion, depending on the presence of a single SNP.

There are several issues in GWAS studies that are more complex than in standard linear regression. First of all, as the number of SNPs (m) outweighs the number of test strains (here, \( n = 38 \)) one has to correct for multiple testing. If a standard 5% significance threshold per
The three identified populations in Fig. 2 result from a principal coordinate analysis of the 38 strains, with 12.4% of the variance explained by the first axis, and 8.9% by the second axis. A way to check this is by calculating the similarity matrix between the strains. For each pair of strains, the fraction of SNPs that are the same is calculated, resulting in an $n \times n$ similarity matrix $K$.

The similarity matrix may be used to correct for population structure (Malosetti et al., 2007; Patterson et al., 2006; Yu et al., 2005). A solution that is often used is a mixed effect model approach:

$$y_i = \mu + \beta x_i + g_i + e_i,$$

where the parameter $g$ is the group effect, and $z_i$ indicates whether strain $i$ is in group A or B. This correction for group effect one can test for SNP effects along the genome, i.e. testing for each SNP the significance of parameter $\beta$. A similar approach can be used in situations where there are strains which can be subdivided in several groups (Kraakman et al., 2004; Pritchard et al., 2000).

In many cases, however, there is not such a clear separation into different groups. A way to check this is by calculating the similarity between the strains. For each pair of strains, the fraction of SNPs that have the same marker score can be calculated, resulting in an $n \times n$ similarity matrix $K$.

The similarity matrix may be used to correct for population structure (Malosetti et al., 2007; Patterson et al., 2006; Yu et al., 2005). A solution that is often used is a mixed effect model approach:

$$y_i = \mu + \beta x_i + g_i + e_i,$$

where $(G_1, G_2, \ldots, G_n)$ follows a multinormal distribution, $G_i \sim N(0, \sigma^2 G K)$, and $\sigma^2 G$ is the genetic variance. This model is used to test for the significance of the SNP effects, $\beta$, along the genome.

4. Results

The fractional adhesion of the STEC O157 strains to Caco-2 cells is highly variable with a frequency distribution resembling an overall skewed distribution (average 0.16, median 0.11). Higher fractions are more rare and concentrated in the human strains (Fig. 1). Mapping of the individual sequences to the reference genome resulted in the identification of 27,980 SNPs among the total set of 38 test strains. When the totality of identified SNPs was used to infer the population structure, at least three separate populations could be identified (Fig. 2). Fig. 2 shows the principal coordinate plot of the 38 strains, with 12.4% of the variance explained by the first axis, and 8.9% by the second axis. The three identified populations in Fig. 2 reflect the LSPA lineages within STEC O157 (IV, III and II) as described by Franz et al. (2012).

After application of the most basic linear regression model (without correcting for population structure), 17 SNPs appeared to be significantly associated with increased adhesion to Caco-2 cells (Fig. 3, Table 2). That is, using a MAF $\geq 0.05$ and a significance level $\alpha = 10^{-4}$, 17 positions on the chromosome of the reference strain show a positive linear relation between SNPs identified in the test strains and fractional adhesion to Caco-2 cells with that for the Sakai reference.

Table 3 shows the loci and, if known, information on biological function, associated with the significant SNPs (SNPs having a MAF $\geq 0.05$ and, in bold face, MAF $\geq 0.1$, i.e. ID 8, 9 and 15) as presented in Table 2. As explained above, instead of having any biological relevance, the results in this SNP analysis could also be the product of a type I error. That is, identifying significant association between genotypic (SNP) and phenotypic (fraction adhesion) information, where there is none.

Having said this, the 17 SNPs identified warrant further investigation with respect to their role in virulence and their use as risk markers for hazard identification. Of these 17 SNPs, eight were non-synonymous in protein-coding regions (Table 3). These SNPs change the protein-sequence and thereby potentially the function of the product.

Table 4 shows which test strains were responsible for the significant SNP analyses in the test strains compared to the Sakai reference for the 17 SNPs identified with $\alpha = 10^{-4}$. Setting the MAF threshold at 0.05 will result in a significant effect when two (or more) test strains appear to share a SNP and are associated with a relatively high (or low) fraction of attachment compared to the test strains that do not differ from the reference Sakai strain for that
site. The result of a type I error in multiple testing (here 27,980 tests) and/or not correcting for population structure may be the cause of this effect.

When correcting for population structure one SNP appears to have a significant effect in this GWAS (Fig. 4). The identified SNP position is 3,115,507 which is close to ID 11 in Table 3 and also has an intergenic position, which in many cases might be irrelevant, but could still be related to promoter sequences.

5. Discussion

Microbiological risk assessment is intended to support decision making in the farm to fork food production chain. Currently food safety criteria are implemented at many levels down to serovar/serotype level for some pathogens (e.g. absence of Salmonella Typhimurium and Salmonella Enteritidis in fresh poultry or STEC O157, O26, O103, O111, O145 and O104:H4 in sprouts). From this perspective it is difficult to assess how the increasing amount of new (molecular) data will influence decision making. Does a 'new' genotype identify a new hazard and thus require a change in policy? And, how should the presence/absence of a virulence gene influence the hazard characterization? There are many such questions and probably even more possible answers related to this problem which can, in general, be referred to as disaggregation. For example, if for a previous ‘generalized’ pathogen (e.g. a serotype) additional information can be specified to describe two ‘less general’ pathogens there is an increase in the number of hazards. This increase

![Fig. 2. Principal coordinate plot of the similarity matrix K. For each pair of strains the similarity was calculated as the fraction of SNPs that have the same score. Black, blue and red dots represent lineage I, I/II and II STEC O157 strains respectively.](image)

![Fig. 3. GWAS plot for trait fractional adhesion to Caco-2 cells. In this plot there is no correction for population structure, i.e. highly overestimating the number of true significant SNP effects.](image)

<table>
<thead>
<tr>
<th>ID</th>
<th>Position (bp)</th>
<th>MAF</th>
<th>β</th>
<th>−log10 (P)</th>
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<tbody>
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<td>808,227</td>
<td>0.05</td>
<td>0.31</td>
<td>7.55</td>
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<td>1,204,977</td>
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<td>0.20</td>
<td>4.01</td>
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<td>3</td>
<td>1,265,758</td>
<td>0.05</td>
<td>0.31</td>
<td>7.55</td>
</tr>
<tr>
<td>4</td>
<td>1,265,760</td>
<td>0.05</td>
<td>0.31</td>
<td>7.55</td>
</tr>
<tr>
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<td>0.08</td>
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<tr>
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<tr>
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</tr>
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<td>0.22</td>
<td>5.29</td>
</tr>
<tr>
<td>11</td>
<td>3,115,509</td>
<td>0.08</td>
<td>0.21</td>
<td>4.64</td>
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<td>0.31</td>
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<td>0.20</td>
<td>4.14</td>
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<tr>
<td>15</td>
<td>4,929,010</td>
<td>0.11</td>
<td>0.23</td>
<td>7.83</td>
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<tr>
<td>16</td>
<td>5,054,140</td>
<td>0.05</td>
<td>0.32</td>
<td>8.47</td>
</tr>
<tr>
<td>17</td>
<td>5,409,931</td>
<td>0.08</td>
<td>0.21</td>
<td>4.64</td>
</tr>
</tbody>
</table>
is exponential because all possible combinations may be relevant. Obviously, when this argument is stretched to the full sequence specification, the disaggregation catastrophe is clear. There will never be enough risk assessments to quantify and predict the full spectrum of all risks. On the other hand, two different subtypes of a ‘generalized’ pathogen may have identical virulence, and their distinction is irrelevant for public health. So, an ‘organization principle’ as a basis for priority setting of highly pathogenic strains is a necessary prerequisite for risk assessment developed from WGS information. This is why linking genotypic with phenotypic properties is essential as it will help to identify high risk isolates from the full spectrum of strains obtained by WGS. In addition, methods for clustering may further reduce the total number of hazards to manageable proportions, for both risk scientists and risk managers. A decisive factor in specifying the clustering level of WGS information is having a good definition of the statement of purpose for the risk analysis. This and insight in the main biological process underlying the risk (e.g. surviving process conditions, the time to initiate for the risk analysis. This and insight in the main biological process information is having a good definition of the statement of purpose for the risk analysis. This and insight in the main biological process information is having a good definition of the statement of purpose for the risk analysis.

### Table 3

Biological information regarding the 17 significant SNPs, obtained using a model without correction for population structure (in Table 2); locus on the reference genome (position (bp)), function of this locus and description of the SNP.

<table>
<thead>
<tr>
<th>ID</th>
<th>Position (bp)</th>
<th>Locus tag</th>
<th>Function</th>
<th>SNP descriptiona</th>
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<tbody>
<tr>
<td>1</td>
<td>108,227</td>
<td>EC0729</td>
<td>RhsC protein</td>
<td>Synonymous; C219T</td>
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<td>2</td>
<td>1,204,977</td>
<td>EC1121</td>
<td>Prophage CP-933R tail fiber protein; putative host specificity protein</td>
<td>Synonymous; C1741T</td>
</tr>
<tr>
<td>3</td>
<td>1,265,758</td>
<td>EC1203</td>
<td>Antitermination protein Q</td>
<td>Synonymous; C12T</td>
</tr>
<tr>
<td>4</td>
<td>1,265,760</td>
<td>non-prophage CP-933R</td>
<td>Encoded by prophage CP-933R</td>
<td>non-synonymous; G14A (R5Q)</td>
</tr>
<tr>
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<td>1,955,401</td>
<td>EC1977</td>
<td>Phage capsid and scaffold protein</td>
<td>Synonymous; C156T</td>
</tr>
<tr>
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<td>1,967,016</td>
<td>EC1987</td>
<td>Tail assembly protein</td>
<td>Synonymous; G351C/T</td>
</tr>
<tr>
<td>7</td>
<td>1,965,259</td>
<td>EC1990</td>
<td>Prophage CP-933 V tail fiber protein; putative host specificity protein</td>
<td>Synonymous; C1062T</td>
</tr>
<tr>
<td>8</td>
<td>2,168,378</td>
<td>EC2164</td>
<td>Minor tail protein encoded by prophage CP-933P</td>
<td>non-synonymous; T424C, C425A (S142E)</td>
</tr>
<tr>
<td>9</td>
<td>2,168,379</td>
<td>Prophage CP-933O</td>
<td>Phage CP-933O</td>
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<tr>
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<td>l-Arabinose 1-dehydrogenase</td>
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<tr>
<td>11</td>
<td>3,115,509</td>
<td>Intergenic</td>
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<td>3,486,443</td>
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<td>EC5283</td>
<td>DNA-binding transcriptional repressor UxuR</td>
<td>non-synonymous; C544A (N178K)</td>
</tr>
</tbody>
</table>

Note:
SNPs having a MAF ≥ 0.05 and, in bold face, MAF ≥ 0.1, i.e. ID 8, 9 and 15.

*SNPs are displayed by type and position in the locus, followed in parentheses by the effect on the amino acid sequence in case of a non-synonymous SNP.

### Table 4

Test strains (second row) causing a ‘significant’ effect (MAF ≥ 0.05 in Table 3) with accompanying fractional adhesion to Caco-2 cells (first row), e.g. for ID 1 the contrast of strains H24 and H19 compared to all 36 other strains gives a significant effect. For each SNP, only the major frequent allele is shown, the empty cells refer to the minor frequent allele.

<table>
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5.1 Biological relevance of identified SNPs

A potential biological relevance can be ascribed to the eight non-synonymous SNPs that are significantly (MAF ≥ 0.05 and a significance level, α = 10−4, associated with higher in vitro adherence to the epithelial cells using the simple linear regression model (Table 3). ECs2332...
encodes for a l-arabinose 1-dehydrogenase (family of oxidoreductases). l-Arabinoose is generally well utilized as a sole carbon source by E. coli O157 strains (Franz et al., 2011) and the intestinal niche occupied by pathogenic E. coli O157 strain EDL933 has been shown to be largely defined by the utilization of arabinoose for colonization (Fabich et al., 2008; Malin et al., 2013). Phenotypic characterization revealed that mutations in the stringent response system resulted in defective utilization of l-arabinose (Oh and Cho, 2014). The ECS5283 locus represents the DNA-binding transcriptional repressor UxuR which plays a role in the D-glucuronate metabolism of E. coli and has been shown to be necessary for maximum ability of E. coli to colonize the intestine (Chang et al., 2004). ECS4969 is a putative phage portal protein, which forms a key component during viral chromosome packaging. Although the same locus was found to be significantly upregulated upon exposure of E. coli O157 to apple juice (Bergholz et al., 2009) the role in pathogenicity remains elusive. Sequence divergence of the phage borne antitermination gene Q (ECS1203), located upstream of stx2, has been associated with variation in transcription of stx2 and with a nonrandom distribution among bovine and human isolates (Franz et al., 2012; Lejeune et al., 2004). The same locus was shown to be significantly up-regulated in a lineage (clade 8) of E. coli O157:H7 commonly associated with human infections (Abu-Ali et al., 2010). Recently, Xu et al. (2012) proposed a model in which Stx2 promotes epithelial cell colonization. Since the antitermination gene Q controls the level of Stx2 production, this gene is a potential candidate for an increased attachment marker. The rhs genes are rearrangement hot spots within E. coli and appear to be under strong positive selection (Petersen et al., 2007). Their extracellular nature may result in a strong positive selective pressure from the host’s immune system and may therefore be involved in O157 host specificity (Liu et al., 2009). They have been reported to promote intestinal colonization of calves (van Diemen et al., 2005).

Bioinformatic analyses support the conclusion that bacterial rhs proteins commonly carry toxin domains and it is proposed that contact-dependent growth inhibition is the primary function of these proteins (Hayes et al., 2014). However, Rhs also appears to coordinate multicellular behavior and biofilm formation (Poole et al., 2011). Interestingly however is the fact that sequence diversity in rhs is also linked to the differentiation of STEC O157 into different clades which in turn have different associations with epidemiology (Liu et al., 2009; Manning et al., 2008). The rhs should be further investigated with respect to combined relevance for biological functioning, phylogeny and epidemiological relevance.

5.2. Advantages and limitations of the approach

Traditionally, STEC hazard identification is based on the seropathotype (SPT) concept of classifying STEC serogroups into different risk classes based on their epidemiological relevance (i.e. severity of disease and involvement in outbreaks) (Karmali et al., 2003). Although informative as an ex post facto determinant of virulence potential, the dynamic nature of STEC virulence in time and place exposes a limitation of SPT classification as a predictive indicator of microbial risk. The approach described here, matching in vitro adherence to epithelial cells as a proxy for virulence with SNP data offers a standardized, reproducible, serogroup independent method for identifying potential candidate genes to be included in epidemiological association studies or more refined hazard identification. Additionally the analysis does not become rapidly unmanageable as more strains are included. SNP analysis of a broad spectrum of isolates (from different sources that are not necessarily associated with human cases) may lead to a less biased association between genotypic and phenotypic strain characteristics. Still, the identification of significant SNPs associated with in vitro virulence should be treated with some caution for several reasons.

First, the in vitro adherence of O157 to human epithelial cells was used as a proxy for in vivo virulence. However, it should be noted that adherence to epithelial cells is only one aspect of the etiology of O157 infection in humans. Although the production of Shiga toxins is the main virulence factor responsible for the more severe symptoms, adherence to the gut epithelium is an important first step in the etiology of STEC infections. The production of Shiga toxins (on expression level, produced toxin level, and/or effect on Vero cells) could be added to this framework and can be analyzed in the same way (with a focus on the prophage regions). Second, SNP analysis based on comparing test strains with one reference strain (here, E. coli O157 strain Sakai) cannot identify SNPs that might be of relevance for hazard identification, but have sequences that are not present in the reference strain. Reference to the pan genome of STEC O157 or even STEC in general could account for this omission (Laing et al., 2010). Alternatively, a “gene-by-gene” approach could be adopted in which the presence/absence of all genes is scored as well as the allelic variation within each gene (Maiden et al., 2013).

Third, instead of having any biological relevance, the results in this SNP analysis could also be the product of a type I error. That is, identifying a false positive association between genotypic (SNP) and phenotypic (fractional adhesion) information, where there is none. This problem will often occur in SNP analysis for MRA where the number of SNPs usually outweighs the number of phenotypic observations. Setting the MAF threshold to a higher level, e.g. 0.1, could alleviate the problem of the small population size. Table 2 shows that ‘only’ three positions on the chromosome of Sakai will be identified as being significant when a MAF ≥ 0.1 would be applied in this study. These represent two loci on the reference genome, i.e. ECS2164 and ECS4864 associated with ‘minor tail protein encoded by prophage CP-9330’ and ‘Rhs protein’ respectively (Table 3). These are, however, non-synonymous SNPs. A further correction for population structure reveals one significant SNP position (3,115,507 which is intergenic) of potential relevance for further biological investigation.

Third, although the loci in which SNPs occur are potentially candidate marker genes for increased adherence to epithelial cells (and maybe virulence in general), the molecular postulates have to be fulfilled in order to prove causal relations (Falkow, 1988).

Finally, the presence of a biomarker (e.g. SNP, gene, metabolite, protein) may by itself not always be a good predictor for risk, since the expression is influenced by a large variety of (dependent biological) factors.
Beside practical implications, this case study also gives insight into which basic statistical elements need to be considered before an association can be a subject for further analysis. That is,

1. The power for analysis needs to be sufficient in order to be able to detect an effect.
2. An optimal number of strains need to be established to get a representative view of the whole (molecular) population.
3. One should correct for population structure before proceeding with further molecular data analysis.

For now, the number of strain samples is very limited for GWAS purposes, and the aim is to continue combining not outbreak biased genotypic with phenotypic STEC O157 data to build a valid statistical model.

Biological confirmation is an essential step before ‘significant’ SNPs can be identified as the cause of hazardous strains. This process consists of,

1. Deletion and complementation studies (according to the principle of Koch’s molecular postulates) to establish the causal relationships.
2. Quantification and calibration of how many differences (e.g. SNP variants) between genomes will lead to treating them as separate categories needs to be established.
3. Reproducibility of the analysis (both experimental and statistical) is a prerequisite for assigning any SNP with a biological relevance. This involves both biological and technical replicates to address variability in the phenotypic response during the statistical analysis.
4. Linking epidemiological data to show the study how disease outcomes following infection are determined by the pathogen by host factors. Human cases caused by any of the isolates with known phenotype can be used to study the association between phenotype (e.g. adherence to the gut epithelium, and growth rate) and disease outcome in humans. The inclusion of outbreak strains facilitates this construction.

Finally, applying the outcomes of statistical associations (confirmed by reproducible data) to microbiological risk assessment, raises the following issues:

1. Integration. A systems biology approach is probably the best way forward to make a link from single scale in vitro testing to a multiple scale interpretation of effects.
2. Anchoring. Translation of molecular investigations to human health risk is still a challenge.
3. Communication. Current policy strategies (e.g. target setting) are based on serovar/serotype level research. This will only change when (statistically) validated model systems can be applied in the domain of public health food safety.
4. Technology. Although not fully expressed in this study, there is a need for development of ‘open data’ systems to support rapid progression. The increasing generation of high throughput data on a global scale needs a central (sentinel) organization to facilitate comparison of risk relevant outputs from individual investigations.

6. Concluding remarks

The bottleneck in the application of molecular tools for microbiological risk assessment has shifted from data acquisition (costs, time) to data analysis and systems approaches. The inability to draw systematic conclusions from this study to STEC in general, represents a bottleneck in the phenotypic response during the statistical analysis.

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References


