Development of an Assay to Detect *Salmonella enterica* Serovar Senftenberg by qPCR

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ABSTRACT

Salmonella food contamination has become a major public health problem due to ongoing, widespread outbreaks and represents a significant financial burden in many countries. *Salmonella enterica sub. enterica ser. Senftenberg* is ranked in the top ten causes of non-human Salmonella disease and is highly tolerant to desiccation, allowing it to persist in poultry farms and feed mills. The zoonotic nature of S. Senftenberg suggests a potential for this serotype to become significant in human disease. Development of a qPCR assay to detect *S.* Senftenberg is crucial for monitoring contamination rates and to prevent human food-related illness. Our aim was to design a highly sensitive and specific assay for the detection of S. Senftenberg to the exclusion of all other *Salmonella* serotypes and species.

20 *S.* Senftenberg samples were sequenced using the Ion PGM™ Sequencer. Draft genomes were assembled and a bioinformatics analysis was performed to identify conserved regions that are unique to *S.* Senftenberg with low similarity to other *Salmonella* serovars. A candidate qPCR assay was designed using a proprietary bioinformatics tool and evaluated against all available GenBank sequences to ensure specificity. The assay was tested using a diverse exclusion panel comprised of other *Salmonella* serotypes and related pathogens. The assay was determined to be specific for *S.* Senftenberg. Assay sensitivity was evaluated by serially diluting S. Senftenberg DNA to extinction. The assay limit of detection was estimated to be approximately 10 CFU/reaction. In conclusion, our *S.* Senftenberg real-time PCR assay is highly sensitive and is able to distinguish S. Senftenberg from other *Salmonella* serotypes.

MATERIALS AND METHODS

Sequencing: 20 *Salmonella* samples were received from a collaborator and nucleic acid was obtained using the PureLink® Genomic DNA Mini kit. 100 ng of nucleic acid was input into the Ion Xpress™ Plus Fragment Library Kit. The recommended protocol for a 300 bp sequencing reaction was followed. During library preparation, samples were ligated with barcoded adapters, allowing for pooling of multiple samples in a single sequencing reaction. The resulting libraries were quantified using the Ion Library TaqMan® Quantitation Kit, followed by template preparation using the Ion OneTouch™ 200 Template Kit v2 DL and the Ion OneTouch™ instrument and enrichment system. After template enrichment, samples were loaded on an Ion 318™ chip and sequenced on the Ion PGM™ System using the Ion PGM™ Sequencing 300 Kit (640 flows).

Sample Number	Salmonella Serovar	Sample Source	Year
1	Johannesburg or Urbana	Pet Food	2012
2	Montevideo	Pet Food	2012
3	Senftenberg	Pet Food	2012
4	unknown	Pet Food	2012
5	Senftenberg	Pet Food	2012
6	Senftenberg	Pet Food	2012
7	Senftenberg	Pet Food	2012
8	Johannesburg or Urbana	Pet Food	2012
9	Senftenberg	Pet Food	2012
10	Senftenberg	Pet Food	2012
11	Senftenberg	Pet Food	2012
12	Senftenberg	Pet Food	2012
13	Senftenberg	Pet Food	2012
14	Senftenberg	Pet Food	2012
15	Senftenberg	Pet Food	2012
16	Senftenberg	Pet Food	2012
17	Senftenberg	Pet Food	2012
18	Senftenberg	Pet Food	2012
19	Senftenberg	Pet Food	2012
20	Senftenberg	Pet Food	2012

Figure 1. Samples Used for This Study

Twenty samples were received for sequencing. Sample serovars were previously identified by the collaborator using a commercial restriction digest-based strain identification system. Sixteen samples were identified as the serovar *S.* Senftenberg; one sample was *Salmonella enterica sub. enterica* ser. Montevideo; two samples could not be differentiated between *Salmonella enterica sub. enterica* ser. Johannesburg and *Salmonella enterica sub. enterica* ser. Urbana; and one sample was unable to be serotyped. All strains were isolated from pet food in 2012.

Bioinformatic analysis and assay design: Candidate primers and probes for a TaqMan assay were designed from two sets of sequences: 1. Draft genomes assembled from sequence reads and 2. Sequences downloaded from Genbank. Sequence reads were assembled into draft genomes using MIRA (2). The 16 Senftenberg draft genomes were analyzed for conserved regions that were common to all sequenced isolates. 59 such regions were identified, one of which exhibited sufficiently low similarity to other Salmonella when aligned to the NCBI non-redundant nucleotide database (top BLAST hits were to *Salmonella enterica sub. enterica* ser. Typhi and *Salmonella enterica sub. enterica* ser. Paratyphi with 77% nucleotide identity). One assay was designed against this region which perfectly matched all assembled genomes. Additionally, three assays were designed against publicly available sequences. All assays yielded no significant hits to off-target organisms when screened against a comprehensive microbial sequence database.

qPCR: qPCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System and analyzed using the Applied Biosystems Sequence Detection Software v1.4.0. Reactions were performed using the standard conditions for TaqMan® Environmental Master Mix, and contained up to 10,000 copies of genomic DNA per reaction. The analysis settings were: Manual Ct, Threshold = 0.2, Auto Baseline, FAM detector (target), VIC detector (IPC)

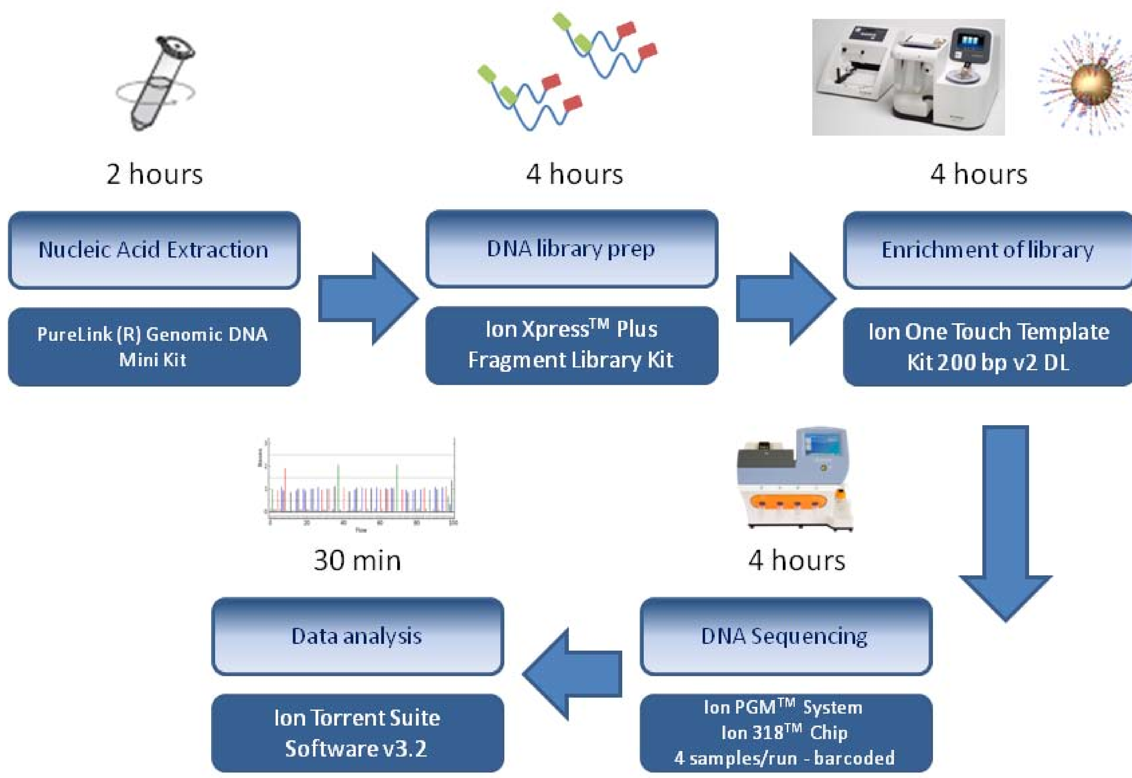

Figure 2. Sequencing Workflow

RESULTS

Sequencing Metric	Min	Max	Average
Read Length	179 bp	244 bp	225.9 bp
Coverage	25.04 X	58.86 X	40.90 X
# Contigs	63	116	83.5
N50	101,830 bp	196,852 bp	128,211 bp

Figure 3. Sequencing Data

The above table shows results from all sequencing runs. Read length ranged from 179-244 base pairs (bp). Sequencing coverage was greater than 25X for all samples resulting in an average of 83.5 contigs for each sample after assembly. The N50 value was calculated for all samples as well. N50, reported in base pairs, is a weighted median statistic such that 50% of the entire assembly is contained in contigs equal to or larger than this value. For all assemblies N50 values were greater than 100,000 bp.

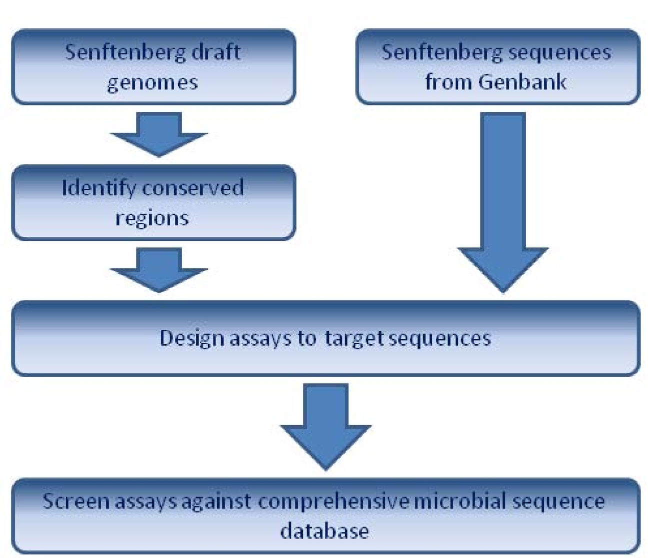

Figure 4. Strategy for design of specific TaqMan® assays

Sequences obtained from the Ion PGM™ sequencer, as well as sequences downloaded from Genbank, were used to design TaqMan® primers and probes which were screened against a comprehensive microbial sequence database.

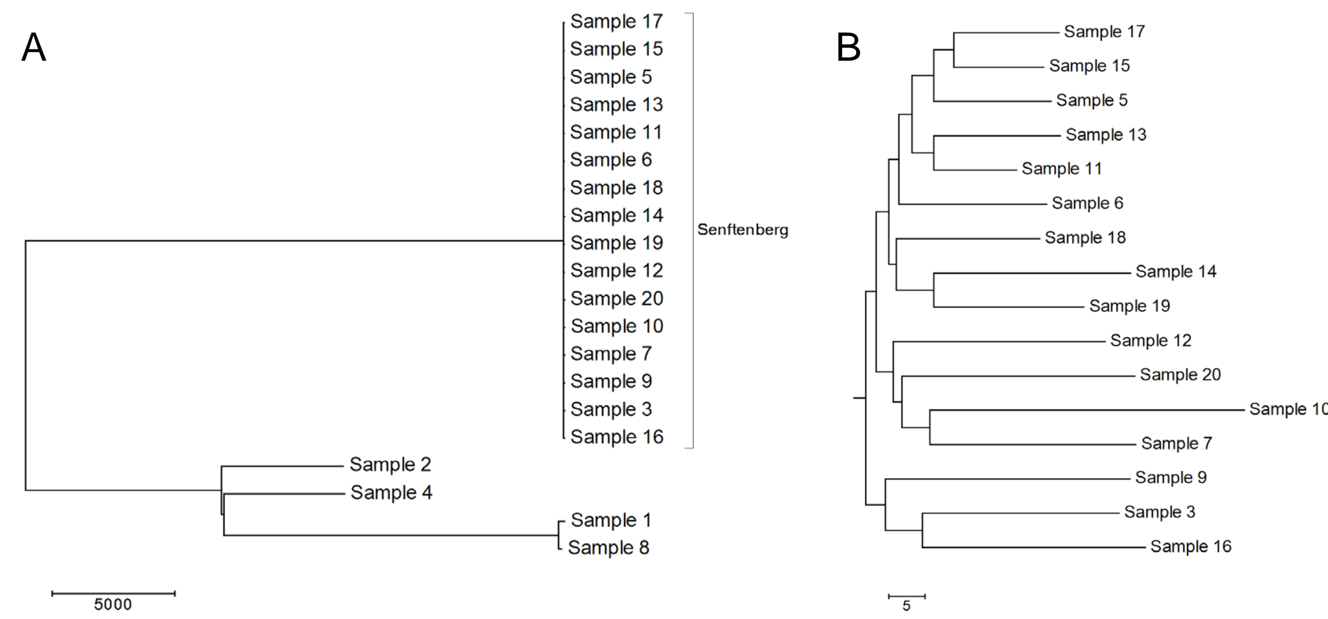

Figure 5. Phylogenetic tree based on SNPs

A) A phylogenetic tree was created to assess genetic relatedness between sequenced samples. The tree was based on 48,792 non-singleton SNPs identified in core regions which were common to all draft genomes. The tree was generated using the neighbor-joining method in MEGA5 (3). B) The subtree consisting only of *S.* Senftenberg samples is also shown.

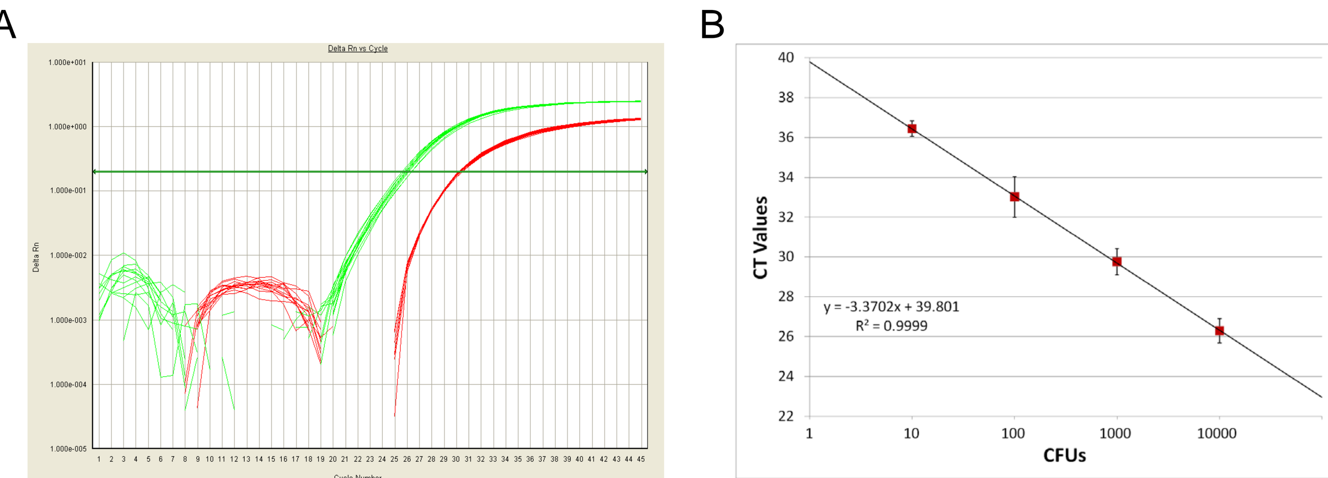

Figure 6. Assay Characteristics

A) qPCR analysis. All 16 confirmed *S.* Senftenberg samples were detected using the assay (green dataset). The assay also includes an internal positive control (IPC) that is used to help gauge the quality of the qPCR data (red dataset). B) Limit of Detection (LOD). The *S.* Senftenberg assay was used to determine the LOD using DNA isolated from the strains supplied by the collaborator. The results are displayed as an average of all 16 strains. For this assay, a CT cutoff of 40 would equate to an LOD of ~1 CFUs detected per reaction. We recommend additional experiments be performed to set an appropriate CT cutoff for each sample matrix of interest.

Inclusivity

Organism	Assay result
S. Senftenberg sample 3	Positive
S. Senftenberg sample 5	Positive
S. Senftenberg sample 6	Positive
S. Senftenberg sample 7	Positive
S. Senftenberg sample 9	Positive
S. Senftenberg sample 10	Positive
S. Senftenberg sample 11	Positive
S. Senftenberg sample 12	Positive
S. Senftenberg sample 13	Positive
S. Senftenberg sample 14	Positive
S. Senftenberg sample 15	Positive
S. Senftenberg sample 16	Positive
S. Senftenberg sample 17	Positive
S. Senftenberg sample 18	Positive
S. Senftenberg sample 19	Positive
S. Senftenberg sample 20	Positive

Exclusivity

Organism	Assay result
Salmonella enterica sub. enterica ser. Albert	Negative
Salmonella enterica sub. enterica ser. Hadar	Negative
Salmonella enterica sub. enterica ser. Heidelberg	Negative
Salmonella enterica sub. enterica ser. Typhimurium	Negative
Campylobacter coli	Negative
Candida albicans	Negative
Cronobacter sakazakii	Negative
Gluconacetobacter liquifaciens	Negative
Pseudomonas aeruginosa	Negative
Staphylococcus aureus	Negative
Vibrio parahaemolyticus	Negative

Figure 7. Specificity

The assay was tested against an exclusion panel of various serotypes of Salmonella and related pathogens and found to be specific for *S.* Senftenberg.

CONCLUSIONS

- All 20 samples were sequenced within 3 days of receiving pure cultures from the collaborator on the Ion PGM™ System.
- Data analysis, including genome assembly and design of specific TaqMan® primers and probes, was completed in eight days.
- Assay development and validation was completed in 10 days.
- The selected assay detects less than 10 CFU per reaction of *S.* Senftenberg genomic DNA.
- The assay is specific for the detection of *S.* Senftenberg and exclusive of other *Salmonella* serovars and non-*Salmonella* organisms.

ACKNOWLEDGEMENTS

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