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Whole genome sequencing used in an industrial context reveals a *Salmonella* laboratory cross-contamination

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ABSTRACT

In 2013, during a routine laboratory analysis performed on food samples, one finished product from a European factory was tested positive for *Salmonella* Hadar. At the same period, one environmental isolate in the same laboratory was serotyped *Salmonella* Hadar. Prior to this event, the laboratory performed a proficiency testing involving a sample spiked with NCTC 9877 *Salmonella* Hadar. The concomitance of *Salmonella* Hadar detection led to the suspicion of a laboratory cross-contamination between the *Salmonella* Hadar isolate used in the laboratory proficiency testing and the *Salmonella* Hadar isolate found on the finished product by the same laboratory. Since the classical phenotypic serotyping method is able to attribute a serotype to *Salmonella* isolates with a common antigenic formula, but cannot differentiate strains of the same serotype within the subspecies, whole genome sequencing was used to test the laboratory cross-contamination hypothesis. Additionally, 12 *Salmonella* Hadar from public databases, available until the time of the event, were included in the whole genome sequencing analysis to better understand the genomic diversity of this serotype in Europe. The outcome of the analysis showed a maximum of ten single nucleotide polymorphisms (SNPs) between the isolates coming from the laboratory and the finished product, and thus confirmed the laboratory cross-contamination. These results combined with all additional investigations done at the factory, allowed to release finished product batches produced and thus circumvented unnecessary food waste and economic losses for the factory.

1. Introduction

Public Health agencies and Food Safety Authorities in America, Europe, Oceania and Asia are routinely using whole genome sequencing (WGS) for surveillance and outbreak investigations to determine if isolates are genetically related (Kan et al., 2018; Rantsiou et al., 2017; Thompson et al., 2017). Conventional typing methods such as Pulsed Field Gel Electrophoresis (PFGE) (Nadon et al., 2017) are now being replaced by this method that provides the highest discriminatory power to differentiate foodborne microorganisms.

This study describes for the first time the application of WGS in an industrial context. We describe the investigation of a potential cross-contamination event of *Salmonella* in a laboratory performing routine microbial analyses.

A finished product produced at a factory located in Europe was found positive for *Salmonella* in 2013 by the laboratory. The *Salmonella* isolate was conventionally serotyped as *S.* Hadar by slide agglutination based on the Kauffmann-White-Le Minor (KW) scheme. At the same period, environmental monitoring in the laboratory indicated a *Salmonella* positive sample from the thermocouple in one incubator. After performing the conventional serotyping on this isolate, it was confirmed to be *S.* Hadar as well. Eight months earlier, a proficiency testing (PT) was completed by this laboratory with NCTC 9877 *S.* Hadar spiked on a sample. Since the PT, no *Salmonella* positives were detected in the laboratory. Although a laboratory cross-contamination was mostly probable, a coincidental contamination of the finished product could not be fully excluded.

S. Hadar is not a rare *Salmonella* serotype in foodborne infections (Hendriksen et al., 2011; Valdezate et al., 2000). Due to the prevalence of *S.* Hadar, a precise typing analysis was required to be able to confirm the hypothesis of the laboratory cross-contamination. In order to differentiate the *S.* Hadar isolates beyond serotype level, WGS was applied on the isolate identified in the finished product, the laboratory environmental isolate, the PT isolate used in the laboratory and the original reference strain used for the PT reordered from the LGC bacterial reference collection.

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Table 1
S. Hadar isolates involved in the suspected laboratory cross-contamination event.

Isolate code	Origin of *S.* Hadar isolate	Isolation date
PIR00616	Proficiency test (PT) sample spiked with strain NCTC 9877	April 2013
PIR00618	Laboratory environmental sample (from the thermocouple in the incubator)	December 2013
PIR00503	Finished product sample (chocolate)	December 2013
PIR00534	Reference strain NCTC 9877, originally used in the PT	Acquired in February 2014[a]

[a] Strain acquired from LGC bacterial reference collection.

2. Materials and methods

2.1. Isolates

Salmonella Hadar isolates involved in the suspected laboratory cross-contamination event are described in Table 1:

2.2. Salmonella detection method

Salmonella was detected according to ISO 6579:2002 with slight modifications. The finished product sample, chocolate, was enriched in 10% non-fat dry milk and incubated at 37 °C ± 1 °C for 18 h ± 2 h. One mL was added to a tube containing 10 mL of RVS medium incubated at 41.5 °C ± 1 °C for 24 h ± 3 h and streaked out on two selective media including XLD. After incubation at 37 °C ± 1 °C for 24 h ± 3 h, typical colonies were confirmed by biochemical and serological testing.

The thermocouple was placed in RVS. After observing growth, isolation and confirmation was carried out as described for the finished product sample.

2.3. DNA extraction

Strains were streaked on Trypticase Soy Agar (TSA) (BioMérieux) to obtain single colonies. After 24 ± 2 h incubation at 37 °C, one colony was used to inoculate 4 mL Brain Heart Infusion (BHI) (Thermo Scientific™, Oxoid™) and was incubated at 37 °C for 8 h. One mL of inoculated BHI was taken and centrifuged (5000 × *g*, 5 min). The pellet was stored at −20 °C until the DNA extraction was performed. For PIR00616, PIR00618 and PIR00503 isolates, the pellet was re-suspended in 160 µL of buffer P1 (Qiagen). Lysozyme (4 mg, Sigma) and RNase A (0.4 mg, Sigma) treatment was carried out. Afterwards, DNA was extracted using the Maxwell RSC cultured Cell kit and the Maxwell Promega Robot (Promega). For PIR00534, DNA extraction was carried out with the QIAamp DNA mini kit (Qiagen) as described in (Portmann et al., 2018). DNA was stored at −20 °C until further analysis. The Qubit™ dsDNA HS Assay kit (Invitrogen) was used to measure the DNA concentration with the Qubit® 2.0 Fluorometer (Invitrogen) according to the supplier's instructions. DNA was standardized to 10 ng/µL with Elution buffer (Qiagen) and stored at −20 °C until used for short read sequencing.

For the PIR00534 isolate, DNA was also prepared for long read sequencing using the Pacific Biosciences system with the purpose of using it as reference for the single nucleotide polymorphism (SNP) analysis. One colony was used to inoculate BHI and grown 24 ± 2 h at 37 °C. Lysis was carried out on the pellet after centrifugation as described above. DNA was extracted using the Maxwell RSC cultured Cell kit and the Maxwell Promega Robot (Promega). DNA was stored at −20 °C until further analysis. The Qubit™ dsDNA BR Assay kit (Invitrogen) was used to measure the DNA concentration with the Qubit® 2.0 Fluorometer (Invitrogen) according to the supplier's instructions. DNA was stored at −20 °C until used for long read sequencing.

2.4. Short read sequencing with MiSeq Illumina

DNA was normalized at 0.2 ng/µL in order to start with 1 ng for the sequencing library preparation using Nextera XT kit following the supplier's instructions (Illumina). A final AMPure beads purification at ratio 0.6× was performed on a Sciclone robotic platform from Perkin Elmer. The quality and quantity of each library were evaluated using a capillary electrophoresis method (LabChip GX Touch from Perkin Elmer). Libraries were pooled based on molarity calculated by the LabChip GX Touch. The equimolar pool was assembled using a Hamilton robotic platform.

The sequencing was performed on MiSeq instrument (Illumina) using v3 chemistry for a 2 × 250 cycles run. The pool was spiked with 2% PhiX and loaded at 8 pM.

All sequences have been submitted to the National Center for Biotechnology Information (NCBI) in BioProject: PRJNA420913 and all sequence read archive numbers (SRR) are available (PIR00503: SRR8649081, PIR00534: SRR8649082, PIR00616: SRR8649083 and PIR00618: SRR8649084).

2.5. Long read sequencing with Pacific Biosciences

High molecular weight DNA was sheared with g-TUBE (Covaris) to obtain around 20 kb DNA fragments. After shearing, the DNA size distribution was checked using the Fragment Analyzer (Advanced Analytical).

DNA was quantified with the Qubit system (Qubit 2.0 Fluorometer and dsDNA Assay HS) and around 5 µg of the sheared DNA was used to prepare a SMRTbell library following the protocol *Procedure and Checklist − 20 kb Template Preparation Using BluePippin™ Size-Selection System* (Pacific Biosciences). The library was size selected at 4 kb with a BluePippin system (Sage Science).

The library was sequenced with R*SII* platform (Pacific Biosciences) on one SMRT cells with P6-C4 chemistry and MagBeads loading (Pacific Biosciences). Sequencing time was 6 h.

The PIR00534 genome was built with a *de novo* assembly carried out with the PacBio Hierarchical Genome Assembly Process (HGAP) version 3 (Pacific Biosciences), closure of the genome by Circlator (Hunt et al., 2015) and final polishing by Quiver v1 (Pacific Biosciences). The total length of the genome assembly was 4,828,940 bp. It was composed of one chromosome assembled and one plasmid.

The NCBI accession number of the PacBio genome is PKPG00000000.

2.6. Bioinformatics analysis

2.6.1. Raw data quality check

In order to evaluate the sequencing run quality, fastq files were assessed with the FastQC software (v0.11.5). Only genomes passing the “per base sequence quality” FASTQC threshold were considered for further analysis.

2.6.2. Salmonella serotyping by whole genome sequencing

The SeqSero software V1 was used to predict the serovar of the *Salmonella* genomes (Zhang et al., 2015).

Table 2
Publically available *Salmonella* Hadar isolates from the NCBI pathogen tree used in the SNP analysis of *S.* Hadar laboratory isolates.

NCBI SRA number	Center name/collected by	Collection date	Geographical location name	Isolation source
SRR4011091	CFSAN/Food and Environment Research Agency(FERA)	2006/2008	United Kingdom	Retail meat
SRR6159201	CFSAN FDA	2009	Germany	Mullein leaves (*Folia verbasci*)
SRR1645752	Public Health England (PHE)	2012	United Kingdom: London	Human
SRR1646058	PHE	2012	United Kingdom: Midlands and East of England	Human
SRR1645282	PHE	2012	United Kingdom: North of England	Human
SRR1645808	PHE	2012	United Kingdom: North of England	Human
SRR1645722	PHE	2012	United Kingdom: Wales	Human
SRR1645209	PHE	2013	United Kingdom: London	Human
SRR1646247	PHE	2013	United Kingdom: London	Human
SRR1646117	PHE	2013	United Kingdom: Midlands and East of England	Human
SRR1645850	PHE	2013	United Kingdom: South of England	Animal
SRR1646279	PHE	2013	United Kingdom: South of England	Human

2.6.3. Single nucleotide polymorphism analysis

High quality SNP (hqSNP) pipeline developed by the Center for Food Safety and Applied Nutrition at FDA (CFSAN SNP Pipeline v.1.0.0) was used for SNP calling on *S.* Hadar isolates (Davis et al., 2015).

2.6.4. Phylogenetic analysis

Maximum-likelihood phylogenetic tree was built with GARLI (Version 2.01.1067) on the SNP analysis results and the tree was displayed with Figtree (version 1.4.3) (http://tree.bio.ed.ac.uk/software/figtree/). The phylogenetic tree shown in this paper was midpoint rooted.

2.6.5. Publically available S. Hadar isolates included in the SNP analysis of S. Hadar laboratory isolates to enable contextual information

In addition to the four *S.* Hadar isolates from the suspected laboratory cross-contamination event, publically available raw sequencing data from *S.* Hadar were selected from the NCBI pathogen tree (www.ncbi.nlm.nih.gov/pathogens) to understand the genetic diversity within the *S.* Hadar (Table 2). This contextual information allowed correct interpretation of SNP differences between the isolates of interest. The objective was to include in the analysis all *S.* Hadar sequenced isolates from Europe that were publically available at the time of the analysis in order to get a sense of the genetic diversity of the *S.* Hadar genomes until 2014 in Europe. A total of 12 public *Salmonella* Hadar sequenced isolates, collected until the time of the suspected laboratory cross-contamination event and sampled in Europe, were added to the analysis. From the 12 available genomic sequence data, nine originated from humans, one from animal, one from mullein leaves (*Folia verbasci*) and one from retail meat.

3. Results and discussion

This study describes for the first time the application of WGS in an industrial context. As part of food safety management systems in food production facilities, factories have an environmental monitoring and product testing program in place to detect early-on deviations in the food production area and their surroundings. Samples taken in this context are sent to laboratories for microbial analyses to control microbiological quality and safety.

In 2013, during a routine microbial testing, a laboratory found a *S.* Hadar positive in a food sample produced at a European factory. The factory immediately retained the finished product batch, initiated extensive cleaning and disinfection, and increased the number of analytical tests. A root cause investigation at the factory was initiated by retrieving all production and analytical data to identify the possible source of the contamination. None of the information collected indicated abnormalities at the production facility. No *Salmonella* was detected in raw materials nor in environmental samples taken at the factory. At the same period, a laboratory environmental sample was found *Salmonella* positive in a thermocouple of the incubator and was as well serotyped as *S.* Hadar. In April 2013, a proficiency testing involving a sample spiked with NCTC 9877 *S.* Hadar was performed in the laboratory that analysed the food sample for the European factory. From the *Salmonella* testing in the laboratory during 2013, only the positives described in Table 1 and the PT in April 2013 were found.

This led to the hypothesis that a probable cross-contamination event in the laboratory has resulted in a false positive result of the food product. Because a coincidental contamination of the finished product with *S.* Hadar could not be excluded, it was a priority to have a confirmation of the laboratory cross-contamination event using a more discriminatory method than the classical phenotypic *Salmonella* serotyping by slide agglutination based on the Kauffmann-White-Le Minor (KW) scheme.

WGS is a powerful method providing the highest precision possible to differentiate foodborne microorganisms and is increasingly being used by Food Safety Authorities worldwide to investigate outbreaks and surveillance (Nadon et al., 2017).

The WGS workflow validated by Portmann and co-workers was applied on the isolates suspected to be implicated in the laboratory cross-contamination event (Portmann et al., 2018). Firstly, the sequence data was evaluated and passed the sequencing quality thresholds. Then, the SNP analysis was performed including all *S.* Hadar genomic data publically available collected before the suspected laboratory cross-contamination event to have an idea about the *S.* Hadar genomic diversity in Europe until 2014. The nine sequenced isolates from humans were submitted by Public Health England (PHE). Due to the lack of isolate submissions by other institutes than PHE, a bias towards clinical isolates from the UK was noticed.

None of the 12 public isolates that were collected until 2014 were genetically similar to the isolates from this study since the 12 genomes differed with > 5700 SNPs compared to the four isolates involved in the suspected laboratory cross-contamination event. The mullein leaves isolate from Germany appeared as the most distant one with > 44,000 SNPs from any other isolate, and was therefore excluded from the analysis (Fig. 1). The 11 isolates from the UK, including 10 clinical and 1 retail meat, could be separated into two main clusters genetically unrelated to the isolates of interest from this study, demonstrating genetic variability within *S.* Hadar in Europe (Fig. 1). One should note that the number of public isolates available at the time of the suspected contamination event was quite small to demonstrate that the strain was not circulating in Europe in 2013.

The SNP analysis showed that the four isolates were genetically very similar with a maximum of 11 SNPs between them suggesting that the isolates shared a common ancestor (Table 3).

The isolate found in the PT sample (PIR00616) in April 2013 and the original PT strain ordered from LGC in February 2014 (PIR00534) differed by seven SNPs. Since both isolates, PIR00534 and PIR00616, originated from the same culture collection, only few genetic differences were expected. The isolate from the laboratory environmental sample (PIR00618) differed by eight SNPs compared to the strain used

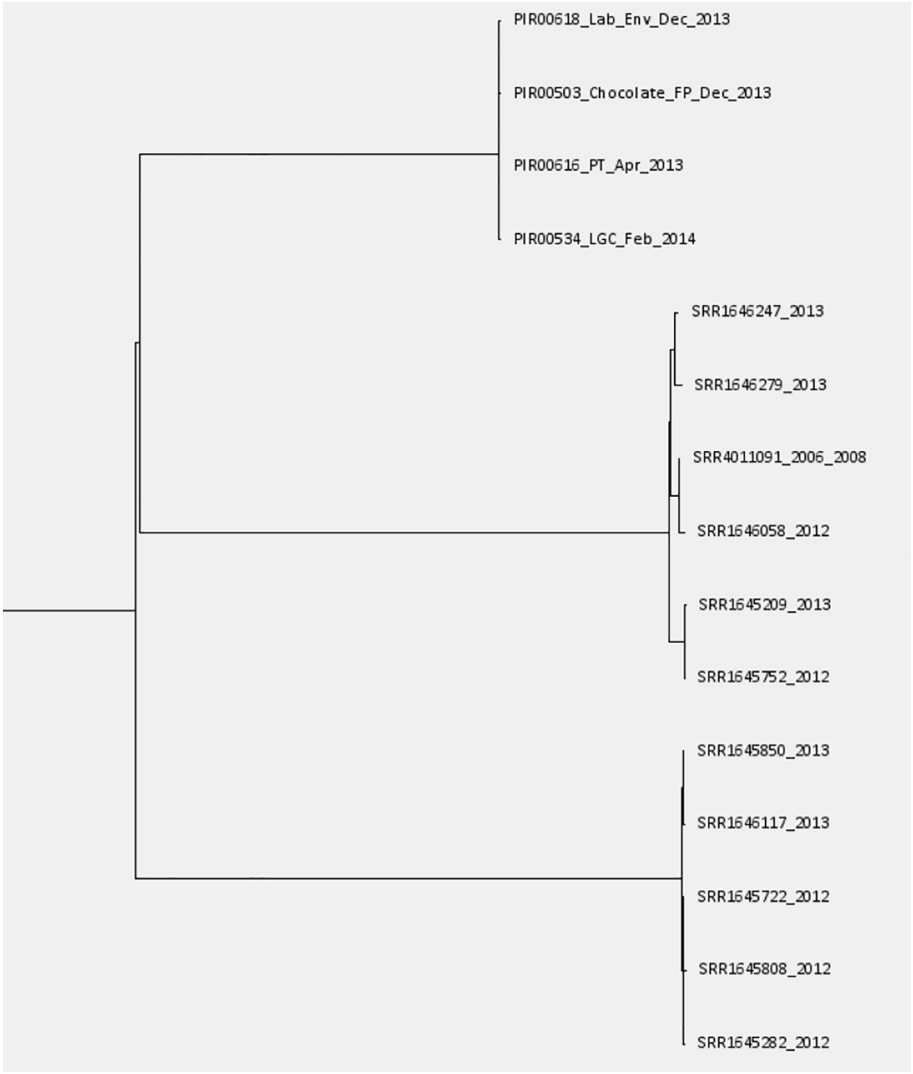

Fig. 1. Maximum-likelihood phylogenetic tree including the four isolates suspected to be implicated in the laboratory cross-contamination event (PIR00503, PIR00616, PIR00618 and PIR00534) and 11 UK publically available *S.* Hadar sequences from the NCBI pathogen tree until 2014.

in the PT (PIR00616). Ten SNPs were identified between the suspected contaminated finished product isolate (PIR00503) and the PT sample (PIR00616), while six SNPs were observed between the finished product isolate (PIR00503) and the laboratory environmental sample isolate (PIR00618).

The fact that a maximum of 11 SNPs were observed between the four *S.* Hadar isolates investigated in this study (PIR00503 and PIR00534) and the large genetic variability observed among the European *S.* Hadar until 2014 (> 5700 SNPs), allowed us to conclude that the PT and the finished product isolates were genetically linked and confirmed the laboratory cross-contamination event. A static SNP threshold cannot be applied to determine if isolates are coming from the same source since many factors such as the organism, time between isolation and exposure conditions of the isolates play a role in the accumulation of SNPs. Pightling et al. (2018) has described criteria when WGS analysis supports a match between two or more genomes. A SNP distance < 21 and a monophyletic tree topology supports a match according to these criteria, which is the case in the genomes analysed in this event.

The WGS outcome combined with all additional investigations done at the factory, allowed to make the decision to release the finished product batches produced and thus circumvented unnecessary food

Table 3
SNP distance matrix between the four *S.* Hadar isolates investigated in this study.

	PIR00503 (FP)	PIR00534 (LGC)	PIR00616 (PT)	PIR00618 (Lab E)
PIR00503 (FP)[a]	0	11	10	6
PIR00534 (LGC)[b]	11	0	7	9
PIR00616 (PT)[c]	10	7	0	8
PIR00618 (Lab E)[d]	6	9	8	0

[a] Finished product sample (chocolate).
[b] Reference strain NCTC 9877 acquired from LGC bacterial reference collection.
[c] Proficiency test sample spiked with the reference strain NCTC 9877.
[d] Laboratory environmental sample (from the thermocouple in the incubator).

waste and economic losses for the factory.

The PT, carried out in April, was responsible for the positive *Salmonella* finding in the chocolate product. The laboratory carried out regular testing for *Salmonella* during 2013 but had only one positive *Salmonella* test during the year, besides the PT in April. Most likely a contamination of the environment in the laboratory occurred while carrying out the PT. In 2013, there was no systematic environmental monitoring put in place and thus the environmental contamination was not noticed. The reason why the isolate stayed dormant for several months and was only detected in December remains unclear. Normally, the thermocouple is not placed in growth media. It is possible that this event allowed the *Salmonella* to enrich to high levels increasing the risk of contamination to the other test samples. After the incident GLP were revised and environmental monitoring was increased in the laboratory.

Rasschaert et al. (2016) reported that laboratory cross-contamination is not a rare event. They used rep-PCR, RAPD and PFGE with two restriction enzymes to confirm a case of a *Salmonella* laboratory cross-contamination (Rasschaert et al., 2016). In their study rep-PCR and RAPD were less discriminatory compared to PFGE that was the gold standard method until recently. Despite demonstrating the usefulness of PFGE to identify laboratory cross-contamination in their example, the authors acknowledged that only sequencing techniques have the discriminatory power to conclude that isolates are identical or highly similar.

A study from De Lappe and co-workers showed that out of 23 probable laboratory cross-contamination incidents, 13 were associated to the laboratory positive control strain, 1 was associated to proficiency test samples and 9 to the other test isolates (De Lappe et al., 2009). This study demonstrated that testing laboratories need to be conscious of this contamination risk notably by positive control strains.

Participation to proficiency testing is important to demonstrate competence while performing specific microbiological examinations (ISO 22117 and ISO 17025). PT and control strains in the laboratory need to be handled with caution to avoid cross-contamination with routine testing samples. Measures to early detect cross-contamination are recommended, such as environmental monitoring and the selection of a rare serotype as a positive control strain. Alternatively, Green Fluorescent Protein (GFP) labelled strains, commercially available, could be an option as GFP can be detected easily under UV light (Ma et al., 2011). Limitations for the use of GFP labelled strains exist since the strains are considered to be Genetically Modified Microorganisms (GMO) and subjected to specific local regulations.

4. Conclusion

The current study showed for the first time one of the benefits of using WGS in an industrial context. *Salmonella* serotyping analysis highlighted a possible laboratory cross-contamination incident that was confirmed by WGS.

Additionally to this application, WGS can offer benefits when being integrated in food safety management systems in the food industry. For instance, WGS can compare isolates taken from samples in the context of environmental monitoring and product testing allowing quality managers to conclude whether the isolates are genetically similar. The WGS outcome is an important piece of information to confirm the root cause of a microbial contamination in a factory. Root cause investigations require an increased sampling and analytical microbial testing. Finding the source of microbial deviations early enough will result in a significant cost reduction.

The WGS analysis is unfortunately still costly not only in terms of infrastructure, equipment and consumables, but also because it requires different kinds of expertise in the fields of in microbiology, sequencing, genomics and bioinformatics. In the future, the decrease of the infrastructure costs and the accessibility of bioinformatics software and genomics training for non-bioinformaticians will benefit the wide use of this methodology in the food industry.

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