

1 Revolutionising Public Health Reference Microbiology 2 using Whole Genome Sequencing: *Salmonella* as an 3 exemplar

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12 ABSTRACT

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14 Advances in whole genome sequencing (WGS) platforms and DNA library preparation have led to the
15 development of methods for high throughput sequencing of bacterial genomes at a relatively low
16 cost (Loman et al. 2012; Medini et al. 2008). WGS offers unprecedented resolution for determining
17 degrees of relatedness between strains of bacterial pathogens and has proven a powerful tool for
18 microbial population studies and epidemiological investigations (Harris et al. 2010; Lienau et al.
19 2011; Holt et al. 2009; Ashton, Peters, et al. 2015). The potential utility of WGS to public health
20 microbiology has been highlighted previously (Köser et al. 2012; Kwong et al. 2013; Reuter et al.
21 2013; Joensen et al. 2014; Nair et al. 2014; Bakker et al. 2014; D’Auria et al. 2014). Here we report,
22 for the first time, the routine use of WGS as the primary test for identification, surveillance and
23 outbreak investigation by a national reference laboratory. We present data on how this has
24 revolutionised public health microbiology for one of the most common bacterial pathogens in the
25 United Kingdom, the *Salmonellae*.

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28 DATA SUMMARY

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- 30 1. PHE Salmonella sequencing data is deposited in the Sequence Read Archive in
31 BioProject PRJNA248792.

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33 **I/We confirm all supporting data, code and protocols have been provided within the
34 article or through supplementary data files. ☒**

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40 IMPACT STATEMENT

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42 The first human genome cost around \$3 billion, and took around 10 years to complete. Advances in
43 DNA sequencing technology (also referred to as whole genome sequencing (WGS)) allow the same
44 feat to be accomplished today for less than \$10000 and less than 2 weeks. This remarkable
45 improvement in technology has also led to a step change in microbiology, increasing our
46 understanding of the evolution of major human pathogens such as *Yersinia pestis*, *Salmonella* Typhi
47 and *Mycobacterium tuberculosis*. While these kinds of academic studies provide unparalleled
48 context for public health action, until now, this approach has not been routinely employed at the
49 frontline. At Public Health England, WGS has been implemented for routine public health
50 identification, characterisation and typing of an important human pathogen, *Salmonella*, replacing
51 methods that have changed little over the last 100 years. Analysis of WGS data has identified
52 outbreaks that were previously undetectable and been used to infer rare antimicrobial resistance
53 patterns. This paper will serve as a notification to the community of the methods PHE are using, and
54 will be of great use to other public health labs considering switching to WGS.

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57 INTRODUCTION

58 Governments have a long history of intervening on behalf of the public health. In the 19th Century
59 public health initiatives sprang up to combat the main afflictions of the day, which were primarily
60 microbiological. In the UK, Edwin Chadwick spearheaded a movement that resulted in the passage of
61 the Public Health Act 1848. One of Chadwick's primary goals was to disperse the 'miasma', or
62 polluted air, that was then held cause diseases such as cholera and chlamydia. This was to be
63 achieved by draining the 30 000 cesspools of London into the river Thames. Unfortunately, the
64 Thames was also the primary source of drinking water for the city at that time. Thus, one of the first
65 modern public health interventions contributed to a dramatic increase in cholera rates in the city
66 (Johnson 2006). While John Snow, Louis Pasteur and Robert Koch soon placed microbiological public
67 health on a firmer footing than 'miasma', the dangers of basing public health action on inaccurate,
68 or out of date, science remain.

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70 Here, we present the experience of a national reference lab that has undergone a transformation
71 from a traditional serotyping laboratory to a state of the art whole genome sequencing laboratory.
72 As we are the first national reference laboratory to do this for *Salmonella*, we believe that relating
73 our experience and approach will be valuable for the wider community.

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77 **Traditional approach to reference microbiology for *Salmonella***
78 **species**

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80 Approximately 10,000 *Salmonella* isolates are referred to the Gastrointestinal Bacterial Reference

81 Unit (GBRU), Public Health England (PHE) each year. Prior to April 2015, all presumptive *Salmonella*

82 isolates received were speciated and sub-speciated using real-time PCR (RT-PCR) (Katie L. Hopkins et

83 al. 2011) and phenotypic arrays (Omnilog). Serological classification, as described in the White-

84 Kauffman-Le Minor scheme (Grimont & Weill 2008), utilises the phenotypic variation seen in

85 flagellar, polysaccharide and capsular antigens, was then used to provide further resolution.

86 *Salmonella* resolves into more than 2600 serotypes according to their antigenic formulae and the

87 procedure relies upon the production of numerous antisera raised in rabbits following a precise

88 immunisation programme. The incidence of different *Salmonella* serotypes identified in the UK is not

89 uniform with only 10 serotypes accounting for approximately 70% of the isolates received by GBRU

90 (Figure 1). Serotyping did not always provide the level of strain discrimination required for outbreak

91 investigation, and was complimented with phage-typing (Callow 1959) and reactive (i.e. not routine

92 for all isolates) molecular methods such as Multi Locus Variable number of tandem repeats Analysis

93 (MLVA) (K. L. Hopkins et al. 2011) or Pulsed-Field Gel Electrophoresis (PFGE) (Peters 2009).

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98 **WGS approach to reference microbiology for *Salmonella* species**

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100 *Genome sequencing*

101 Since April 2015, WGS has been the one procedure performed on all cultures of *Salmonella sp.*

102 referred to GBRU by frontline hospital microbiology laboratories, private laboratories and food,

103 water and environmental laboratories. All other typing methods previously employed have been

104 significantly reduced or withdrawn. On receipt, original cultures are directly inoculated into 750 µl of

105 nutrient broth and incubated overnight at 37°C. DNA extraction is performed using the

106 Qiasymphony automated DNA extractor (Qiagen) and DNA is quantified using the Glomax

107 (Promega). DNA is submitted to the central Genomic Sequencing and Development Unit at PHE,

108 where Illumina Nextera XT DNA libraries are constructed and sequenced using the Illumina HiSeq

109 2500 in fast mode. The samples are then deplexed by Casava software and Trimmomatic (Bolger et

110 al. 2014) used to trim any data with a phred score less than Q30 from the beginning and end of the
111 reads. The outline of this process can be seen in Figure 2.

112

113 *Bacterial identification and serogroup designation*

114 A sample of k-mers (DNA sequences of length k) in the sequence data are compared against the k-
115 mers of 1769 reference genomes representing 59 pathogenic genera obtained from RefSeq. The
116 closest percentage match is identified, and provides initial confirmation of the species and sub-
117 species of *Salmonella*. This step also identifies samples containing more than one species of bacteria
118 (i.e. mixed cultures) and any bacteria misidentified as *Salmonella* by the sending laboratory.

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120 Once the sample is confirmed as *Salmonella*, the sequenced genome is interrogated with sequences
121 in the Multi Locus Sequence Typing (MLST) database (Achtman et al. 2012) using a modified version
122 of SRST (Inouye et al. 2012). This provides a quality assessed sequence type (ST). Achtman *et al* have
123 shown that *Salmonella* serotypes generally belong to clonal complexes of related Sequence Types
124 (ST) known as e-burst groups (EBGs) and have described the correlation between EBGs and serotype
125 (Achtman et al. 2012). At PHE a database containing matched MLST data and phenotypic serotype
126 designation for more than 12000 isolates of *Salmonella* allows us to infer a serogroup from the
127 sequence type with 96% accuracy (Ashton, Nair, et al. 2015). The primary error types are (i) two
128 different serotypes having the same ST, e.g. ST 909 is both *Salmonella* Richmond and *Salmonella*
129 Bareilly (ii) processing errors and (iii) inaccuracy in serotype designation of public data (Ashton, Nair,
130 et al. 2015). The inferred serotype is then reported back to the sending laboratory, along with the ST
131 of the isolate, to maintain backwards compatibility with historical data. This provides customers with
132 a consistent service and facilitates data exchange with Public Health colleagues both locally and
133 internationally, as well as others in the veterinary, food, water and environmental microbiology
134 disciplines.

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136 *Outbreak detection and investigation*

137 Over 70% of isolates received by GBRU belong to the most common 14 serotypes and, as with
138 serotype, ST alone is not discriminatory enough for outbreak detection and public health
139 investigation. Whole genome single nucleotide polymorphism (SNP) typing is performed on samples
140 that belong to the most common e-burst groups. This involves mapping the sequence reads to an
141 appropriate reference genome (within the same e-burst group) using BWA mem (Li & Durbin 2009),
142 before identifying SNPs with GATK (DePristo et al. 2011). High quality SNPs are then stored in a

143 database where they can be queried to generate sequence alignments for input into phylogenetic
144 algorithms.

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146 Within PHE, outbreaks of *Salmonella* were traditionally detected using an exceedance algorithm
147 (Noufaily et al. 2013) based on serotyping and phage typing data. The lack of discrimination
148 associated with these typing techniques results in a high false positive rate of exceedance
149 notifications (Noufaily et al. 2013). In contrast, whole genome SNP typing offers unprecedented
150 resolution in linking cases providing added certainty to outbreak definitions, transmission networks
151 and other aspects of the underlying epidemiology.

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153 A hierarchical ‘SNP address’ approach is employed that groups isolates together into clusters of
154 increasing levels of similarity. The pairwise SNP distance is calculated for each pair of isolates in the
155 analysis set. This distance matrix is then subjected to single linkage clustering at 250, 100, 50, 25, 10,
156 5 and 0 SNPs. The end result is a SNP address that identifies clusters of isolates at each level of the
157 hierarchy (Figure 3). The SNP address approach for identifying epidemiologically significant clusters
158 (i.e. outbreaks) correlates well with existing workflows and is phylogenetically informative, as all
159 isolates are placed into haplotypes derived from a phylogeny of the clonal complex in question.

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161 Median turn around time from the receipt of a sample at the reference laboratory to a reported,
162 WGS based, result over the last 4 weeks before submission of this manuscript (weeks 43-46, 2015)
163 months was 10 working days (David Powell, PHE, personal communication). Once an outbreak has
164 been identified it is important to confirm additional cases quickly in order to expedite
165 epidemiological investigations. To this end we are exploring the use of WGS data to design
166 outbreak/incident-specific RT-PCR or High Resolution Melt assays to screen additional referred
167 cultures as being part of specific outbreaks based on SNPs that are unique to this cluster. Then, in
168 the case of an incident, all samples could be rapidly screened upon receipt to ascertain if they are
169 part of the outbreak. Epidemiological investigation could then proceed in a more timely fashion.
170 These isolates could be sequenced as a matter of urgency using a MinION device (Oxford Nanopore
171 Technologies), for example, which could confirm the isolate as being part of a known outbreak, or
172 not (Quick et al. 2015) (PMID 26025440).

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174 *Further microbiological characterisation*

175 One of the most attractive aspects of WGS is the fact that it lends itself to a ‘single process, multiple
176 tests’ approach. For example, once you have generated the sequence in order to do whole genome

177 SNP typing, the data is available in perpetuity for other tests, e.g. characterisation of virulence and
178 other molecular markers e.g. of antimicrobial resistance. Known antimicrobial resistance
179 determinants can be readily identified from WGS data. We carried out a pilot study to compare
180 genotypic vs phenotypic resistance typing in 642 *Salmonellae* of which 57.5% were susceptible and
181 24.7% MDR. Results showed a greater than 99% success rate (unpublished data) indicating the
182 potential of adding this into the routine *Salmonella* work flow. Phenotypic screening could be used
183 on a small proportion of isolates in order to detect novel or emerging resistance mechanisms. The
184 fact that the data is available in perpetuity allows re-testing when e.g. novel resistance mechanisms
185 emerge.

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187 *Data sharing*

188 The genomic data of all *Salmonella* sequenced at PHE is publically released into the NCBI BioProject
189 PRJNA248792 within two weeks of the sample report date. The prompt release of data is to facilitate
190 international tracking and surveillance of food and waterborne, gastrointestinal pathogens in far-
191 reaching distribution networks (Byrne et al. 2015). Globally, national surveillance organizations, such
192 as the Food and Drug Administration and the United States Centre for Disease Control, are also
193 uploading *Salmonella* genomes from their surveillance activities into public sequence archives.
194 Algorithms that enable the timely and sensitive comparison of these datasets are a high priority, as
195 this will be needed to monitor international patterns in gastrointestinal infection. While meta-data is
196 limited for privacy reasons, publically releasing the data provides a great resource for academic
197 researchers interested in gastrointestinal pathogens.

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199 *Infrastructure requirements and challenges*

200 WGS is obviously a very exciting technology, however it is one that requires substantial investment in
201 infrastructure. Public Health England have invested millions in sequencing and molecular biology
202 hardware, high performance computing and staff costs (wet lab and bioinformaticians) to deliver this
203 service. There are also many challenges involved in changing skillsets and mindsets. Practical
204 implementation and integration across different departments (microbiology, sequencing,
205 bioinformatics and epidemiology) is also a challenge, but necessary to ensure the success of a
206 project such as this.

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210 **Future perspectives**

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212 It is an exciting time to work in microbiology and genomics. Even before the repercussions of one
213 revolution (short read, whole genome sequencing) have had their full impact on reference and
214 clinical microbiology, another revolution (long read, portable sequencing) is on the horizon. Exactly
215 how this second revolution will affect clinical and reference microbiology is currently unclear but
216 two obvious applications present themselves. Firstly, the ability to fully assemble large numbers of
217 genomes could provide a step-change in resolution for determining whether two genomes are
218 related. Currently, it is the core genome that is the focus of the majority of genomic epidemiology.
219 Being able to analyse the entire genome provides opportunities for using the accessory genome to
220 determine how related two isolates are. However, this approach needs to be thoroughly
221 investigated and assessed for sensitivity and specificity as compared with a core genome approach.
222 We need to be mindful that the dynamic accessory genome could be misleading as to the
223 relatedness of isolates (Lauren Cowley, PHE, personal communication). The other application of this
224 new wave of sequencers comes from their portability and low profile infrastructure requirements.
225 Many smaller hospitals and front line laboratories could not justify setting up the infrastructure
226 required to run a large machine as they are unlikely to perform enough sequencing to make it
227 economically viable. However, it would be feasible to employ smaller devices like the MinION and
228 use them to sequence suspect outbreak strains. The data could be streamed to a repository
229 maintained by the reference laboratory where the SNPs identified in the sample could be used to
230 place the sample onto a tree and call it as outbreak or non-outbreak (Figure 4). This approach has
231 already been used in an outbreak situation (Quick et al. 2015). There is a big question for many
232 organisations as to whether to invest heavily now in proven, 'work-horse' machines, or to hold on
233 for exciting new technologies to mature, with the potential increases in throughput and decrease in
234 cost promised by these 'third generation', or 'third revolution' (Loman & Pallen 2015), technologies.

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239 CONCLUSION

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241 WGS for routine public health surveillance offers a host of scientific, technical and economic
242 advantages compared with conventional microbiological methods. A single, highly automatable
243 process provides speciation, identification, typing and characterization to a level at least equivalent,
244 and often superior, to the previous 'gold-standard' techniques. In addition, the data is uniquely
245 portable, allowing rapid comparison with other public health institutions and re-use by the wider
246 scientific community.

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249 ACKNOWLEDGEMENTS

250

251 We would like to acknowledge the contribution of everyone working in the Salmonella
252 Reference Service, the Genome Sequencing and Development Unit and Infectious Disease
253 Informatics at Public Health England, for their invaluable contributions to this work. We
254 would also like to thank Nick Loman for significant contribution toward figure 4.

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257 ABBREVIATIONS

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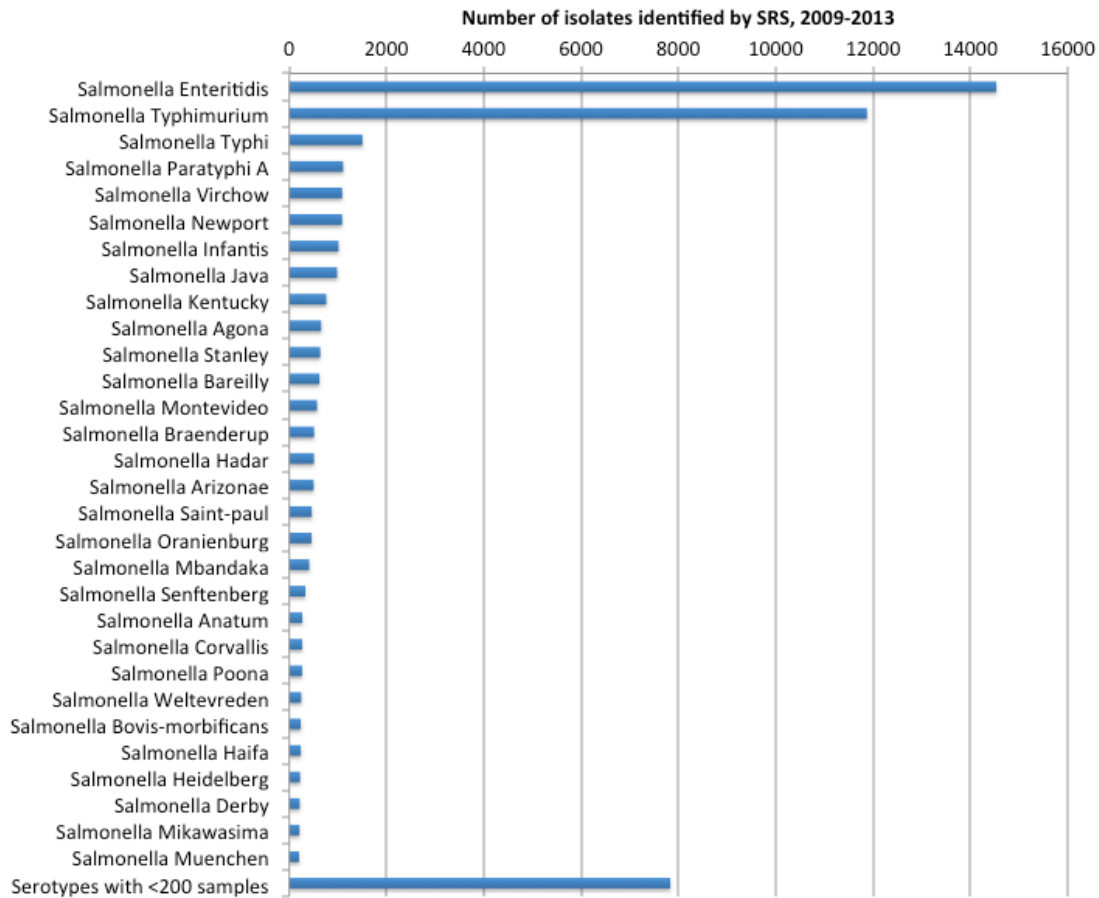
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359 **FIGURES AND TABLES**

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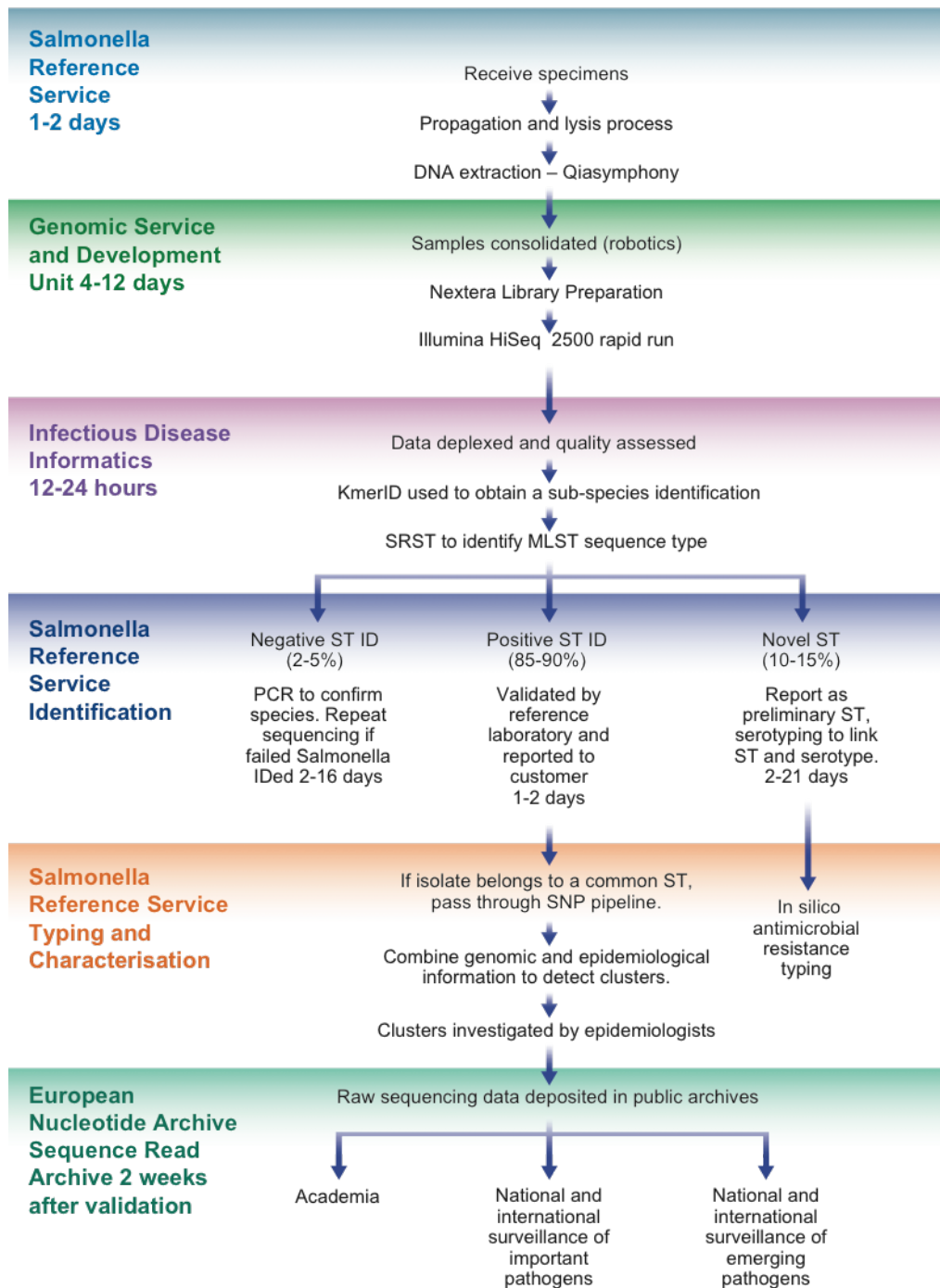
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363 **Figure 1: Serotypes with more than 200 isolates received in the 4 year period 2009-2013 by**

364 **Salmonella Reference Service, Public Health England.**

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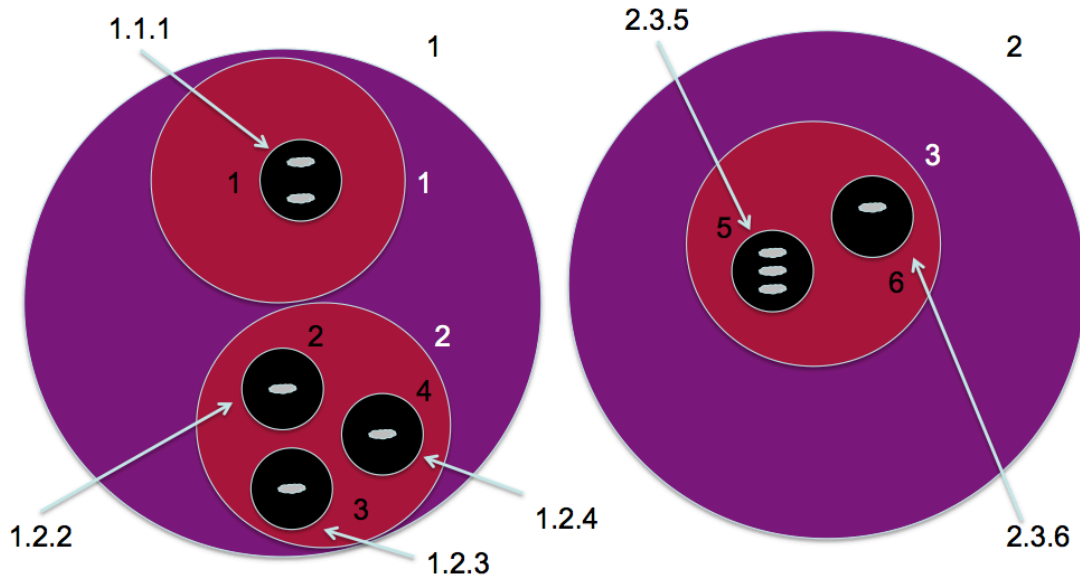


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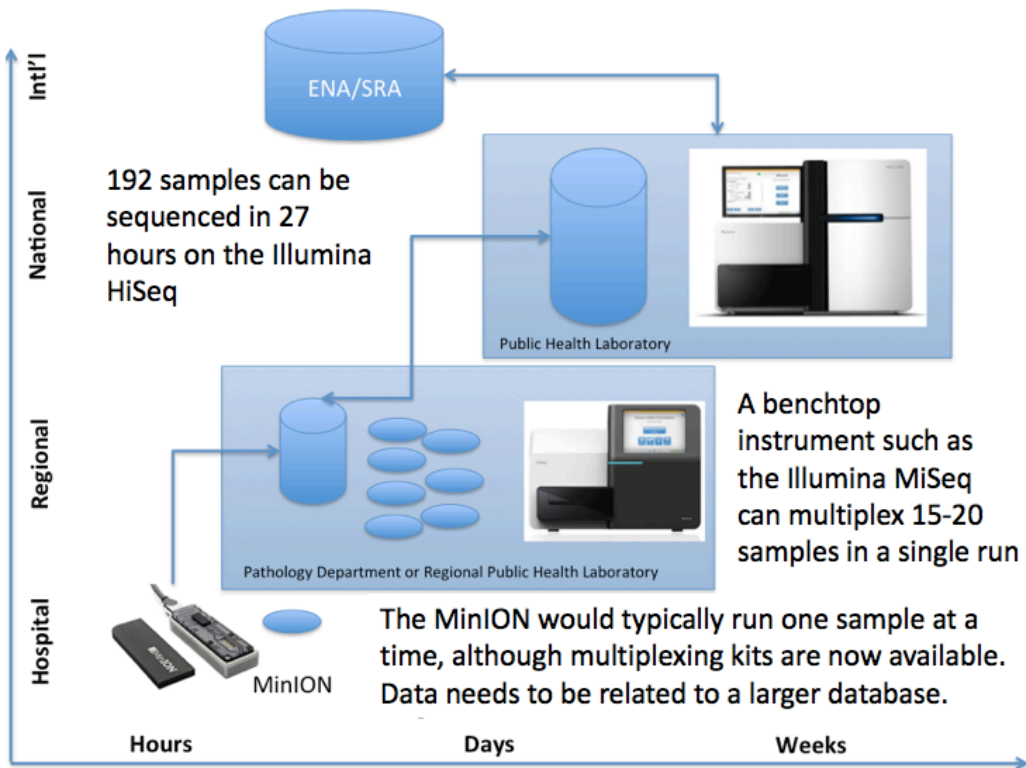
367 **Figure 2: The current workflow for the use of whole genome sequencing by the Public Health**

368 **England Salmonella Reference Service**

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 371 **Figure 3: Schematic of how the SNP address works**
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 374 **Figure 4: Example of future public health workflow**
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