Real-time pathogen surveillance systems using DNA sequencing

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Synopsis

Microbiological research has uncovered the basis of fermentation, infectious disease, vaccination and antibiotics. Now, a technological revolution leveraging DNA, the code of life, has allowed us to unravel cellular and evolutionary processes in exquisite detail. Today our need for new innovation is still great. The modern world is a challenging environment: over-population, climate change and highly mobile populations create a high risk of pandemic disease especially from viruses and many bacteria are now resistant to our life saving antibiotic drugs due to overuse. In hospitals, the spread of pathogens can be rapid and life threatening. Whole-genome sequencing has the power to identify the source of infections and determine whether clusters of cases belong to an outbreak. Portable, real-time nanopore sequencing enables sequencing to be performed near the patient, even in resource-limited settings. Integrating with existing datasets allows digital surveillance able to detect outbreaks earlier while they can still be contained. Early demonstrations of the power of whole-genome sequencing for outbreak surveillance have made it an area of intense interest and further development in laboratory methods and infrastructure will make it an important tool that can be deployed in response to future outbreaks.

Submitted work

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1. Introduction

1.1 The discovery of microbes

The first descriptions of microorganisms were made by Antony van Leeuwenhoek in 1676[1]. Using his homemade microscopes, developed for inspecting the quality of cloth, he observed a menagerie of tiny 'animalcules' in samples of lake water and plaques from his own teeth. He notified the Royal Society, who were initially sceptical, but commissioned Robert Hooke to build a powerful microscope. Hooke successfully reproduced a bizarre experiment Leeuwenhoek had conducted to see if black pepper was covered in points which were responsible for its sharp taste. He mixed ground pepper with water, and days later he discovered it teeming with microbes. The Royal Society were convinced and made him a Fellow and the field of microbiology was born.

1.2 The 'Golden age' of bacteriology

1.2.1 Pasteurisation

Two hundred years later, Louis Pasteur and Robert Koch ushered in the 'Golden Age of Microbiology'. They unravelled many fundamental microbial processes and their relationship to disease during this period. Pasteur wished to understand the microbiology of food spoilage organisms. He discovered the process of alcoholic fermentation by yeast and that contamination by another microorganism could turn wine into vinegar. He devised his method of heat sterilisation which was later routinely

applied to beer and milk[2]. In a famous experiment, he used swan-necked flasks to show if bacteria could not fall into sterile broth then it would not become contaminated. This was strong evidence against the popular theory of spontaneous generation[3] whereby living organisms could arise from inanimate matter.

1.2.3 Discovery of the anthrax bacillus

Robert Koch was a German physician and has been described as the 'father of medical microbiology'. Koch built on Pasteur's work on germ theory in order to confidently attribute diseases to infection by particular microbial species. While investigating anthrax deaths in humans and livestock, Koch demonstrated that the disease could be transferred to healthy animals by inoculating them with blood from diseased animals. Further, the presence of rod-shaped bacteria in the blood was required for disease transmission. He went on to demonstrate the role of spores in anthrax lifecycle and how their presence in contaminated soil could make it infectious for years. He later discovered the microbes responsible for tuberculosis and cholera.

1.2.4 Koch's plate technique

Koch demonstrated the utility of culture in medical microbiology through the use of his plate technique[4]. During his studies on anthrax he noticed that the rods were often elongated and notched and suspected this related to how the cells reproduced. He realised that culture needed to take place on solid media to ensure that cultures were 'pure' i.e. derived from one starting cell. This led him to develop agar media which

bacteria grow well on and stays solid at temperatures needed for incubation. He grew his cultures in a shallow dish with an overhanging lid to prevent contamination invented by his assistant Petri.

1.2.5 Koch's postulates

Based on the techniques he had available to him, Koch devised a set of rigorous guidelines for determining the causality of disease. They required that the pathogen be present in all diseased individuals, be isolated in pure culture and that the pure culture be able to cause disease in a healthy animal model. These would remain unfulfilled if the organism was unculturable or for viral infections until the invention of the electron microscope.

1. Infected tissue must show the presence of a particular microorganism not found in healthy animals.
2. The microorganism must be isolated and grown in a pure culture.
3. When injected into a healthy animal, the microorganism must cause the disease associated with it.
4. This "second generation" microorganism should then be isolated and shown to be identical with the microorganism found in 1.

Figure 1. Koch's postulates for causality of disease reproduced from http://ocp.hul.harvard.edu/contagion/koch.html.

1.3 Antimicrobial compounds

1.3.1 History of early antibiotics

The first effective antimicrobial drug was Salvarsan, an arsenic compound synthesised in Paul Ehrlich's lab. Ehrlich screened many compounds in the hope of finding a 'magic bullet' that was not fatally toxic to the patient. The search paid off in 1909 when his assistant Sahachiro Hata showed he could cure a Guinea pig of a spirillum infection using a compound known as 'Compound 606'. Subsequent human trials showed it was an effective treatment for syphilis and proved to have few side effects. Sulphonamides such as Prontosil introduced in 1932, were the first effective antibiotics that could be taken orally to treat systemic infections. The compound inhibits bacterial growth by blocking folic acid synthesis which is required to make nucleic acids. This led to dozens of related compounds being produced and as the only effective antibiotic available before penicillin it was used to treat many conditions. A related compound, Sulfamethoxazole is still in use today usually in the form of co-trimoxazole for treating urinary tract infections.

Penicillin, one of the most celebrated antibiotics, is an antimicrobial compound produced by the fungus *Penicillium notatum*. After returning from holiday microbiologist Alexander Fleming noticed a mould had contaminated his plates and that there were no staphylococci colonies growing close to it[5]. He realised that the fungus was producing a bactericidal substance and showed it was able to kill a number of other important pathogens. Research into the mass production of penicillin eventually led to

the production of 2.3 million doses of penicillin by the end of the World War II. Penicillin quickly became indispensable and doctors prescribed it for infections of gonorrhoea, streptococci and staphylococci. Fleming had warned about the dangers of misuse of penicillin as he has observed bacteria acquire resistance to it in the laboratory[6], however the use of the miracle drugs grew exponentially. The first penicillin resistant *Staphylococcus aureus* were detected as early as 1947[7] and for all new classes of antibiotics such as aminoglycosides, chloramphenicols and tetracyclines, resistant strains were detected within a few years of each being used[8].

1.4 Phenotypic identification

1.4.1 Microbial identification

Microbes are classified using phenotypic properties including cellular morphology, cellular aggregation and metabolic capability. Morphologies include cocci, bacilli, filamentous and spirochete forms with cocci being subcategorised by arrangement such as diplococci and chains. Staining can make bacteria more visible through a microscope: the most famous being the Gram stain developed by Hans Christian Gram in 1884[9]. The technique involved a primary stain of crystal violet and counterstain of safranin which gave a binary classification of most bacteria, depending on the peptidoglycan content of the cell wall. Many viruses are too small to be seen by a light microscope they could not be seen until the invention of the transmission electron microscope in 1935, however the presence of aggregates e.g. Negri bodies in Rabies infection were used to detect viruses before this.

1.4.2 Biochemical tests

Bacteria can be identified via their metabolic functions using a panel of biochemical tests. For example, the catalase test is used to distinguish *Staphylococcus* species from other aerobic cocci. Catalase producers metabolise hydrogen peroxide into water and oxygen gas which is observed as bubbles. The coagulase test can further identify *Staphylococcus aureus* from other Staphylococcus species. The presence of coagulase mean fibrinogen is converted to insoluble fibrin which turns the liquid solid. These tests are very common in clinical microbiology labs as they are quick and relatively simple. They are commonly automated on machines performing many tests simultaneously on cards such as in the bioMérieux VITEK 2 which can perform automated identification and susceptibility testing.

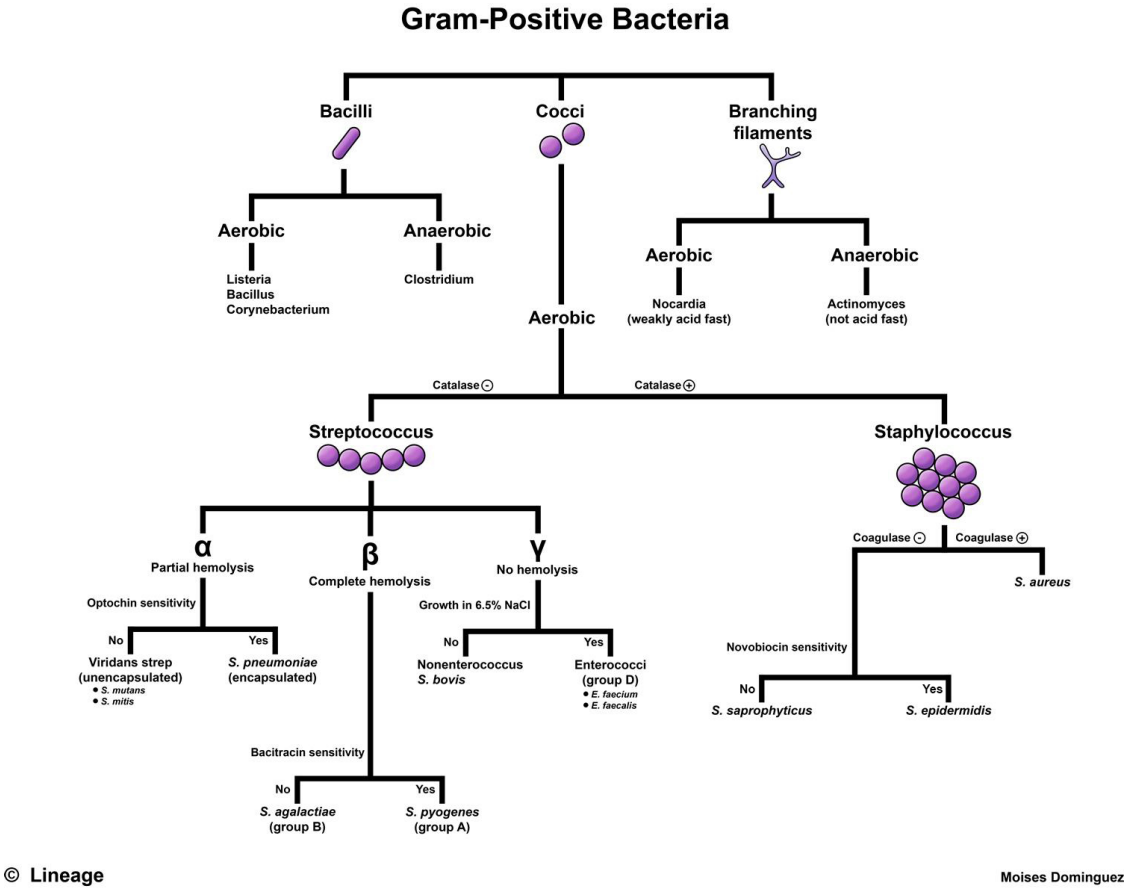

Figure 2. Reproduced from http://step1.medbullets.com/microbiology/104192/gram-positive-bacteria showing how you would identify Gram-positive bacteria such as Staphylococcus aureus using phenotypic methods.

1.4.3 Antibiograms

Bacterial antibiotic susceptibility testing is typically performed using the disc diffusion method. A disc containing a known concentration of antibiotic is added to a Petri dish of growing bacteria. The bacteria will grow until they become inhibited by the antibiotic diffusing from the disc. The size of the inhibition zone indicates effectiveness of the antibiotic at inhibiting bacterial growth. The assay allows multiple antibiotics or concentrations to be tested on the same plate. Antibiotic sensitivity testing is primarily

used for prescribing an effective antibiotic to treat an infection but as patterns in antibiotic sensitivity vary greatly even within a species, patterns of sensitivity or resistance can be used as evidence in epidemiological investigations.

1.4.4 Phage typing

Phage typing uses panels of bacteriophages to discriminate between bacterial strains. A grid is drawn on a plate and different bacteriophage are added to each region. Bacteriophage are highly diverse yet have a narrow host range as they can only infect bacteria with receptors to which they can bind. The pattern of lysis by different bacteriophages provides the phage typing profile. If two isolates produce the same phage pattern that is used as evidence that they are closely-related strains.

1.5 Genotypic identification

Combinations of phenotypic tests have defined the classical microbial taxonomy used in clinical microbiology today. However, phenotypic tests suffer from numerous limitations; they rely on culture so are difficult for slow growing or fastidious organisms e.g. *Mycobacterium tuberculosis*, multiple tests are required to identify each organism and the interpretation of test results can be very subjective[10]. Therefore genotypic tests relying on molecular methods such as the polymerase chain reaction and deoxyribonucleic acid sequencing are gradually replacing phenotypic methods.

1.5.1 Deoxyribonucleic acid (DNA)

DNA and ribonucleic acid (RNA) are polymers made up of nucleobases that store genetic information and are essential to all known life. In 1928, Frederick Griffiths performed an experiment demonstrating that an unidentified transforming principle could induce a change from non-capsular to capsular in *Streptococcus pneumoniae*[11]. The first demonstration that the transforming principle was DNA came from Avery-MacLeod-McCarty experiment in 1944[12]. At the time it was widely assumed that the heritable material would be protein but Avery and colleagues managed to carefully separate DNA from the rest of the cellular components and show that it alone possessed transformative capability. Later, further confirmation that DNA is the transforming substance came in 1952 from Hersey and Chase[13] in an elegant experiment where they labelled T2 phage with radioactive isotopes of either phosphorus or sulphur. The progeny of these phages contained radioactive phorphorus but not radioactive sulphur proving that the genetic material was DNA not protein. The structure of DNA and model for semi-conservative replication was proposed by Crick and Watson in 1953[14] by formulating a model that fitted known chemical properties of nucleic acid and interpretation of a X-ray diffraction photo taken by Rosalind Franklin. Crick later published the 'central dogma of molecular biology'[15] which stated that genetic information flowed from DNA to RNA to protein in general transfers as well as other information flows such as RNA to DNA in special circumstances. Critically, however, once genetic information is encoded in proteins, it cannot transfer back again.

1.5.2 The polymerase chain reaction (PCR)

PCR is a system for the amplification of DNA targets using short oligonucleotide primers. It was a developed by Kary Mullis while working at Cetus Corporation who were developing a diagnostic assay for sickle cell anaemia[16]. Mullis realised that he could modify existing polymerase extension methods by adding a reverse primer on the opposite strand to achieve exponential amplification of a target region. The technique has revolutionised the detection of genetic mutations, the diagnosis of infectious disease, forensics and research. The components of the reaction are DNA template, forward and reverse primers, thermostable polymerase and deoxynucleotide triphosphates (dNTPs). Products of PCR are usually visualised using agarose gel electrophoresis, a technique used to separate DNA molecules by size; visualising a band on a gel of the expected size is indicative of a positive PCR.

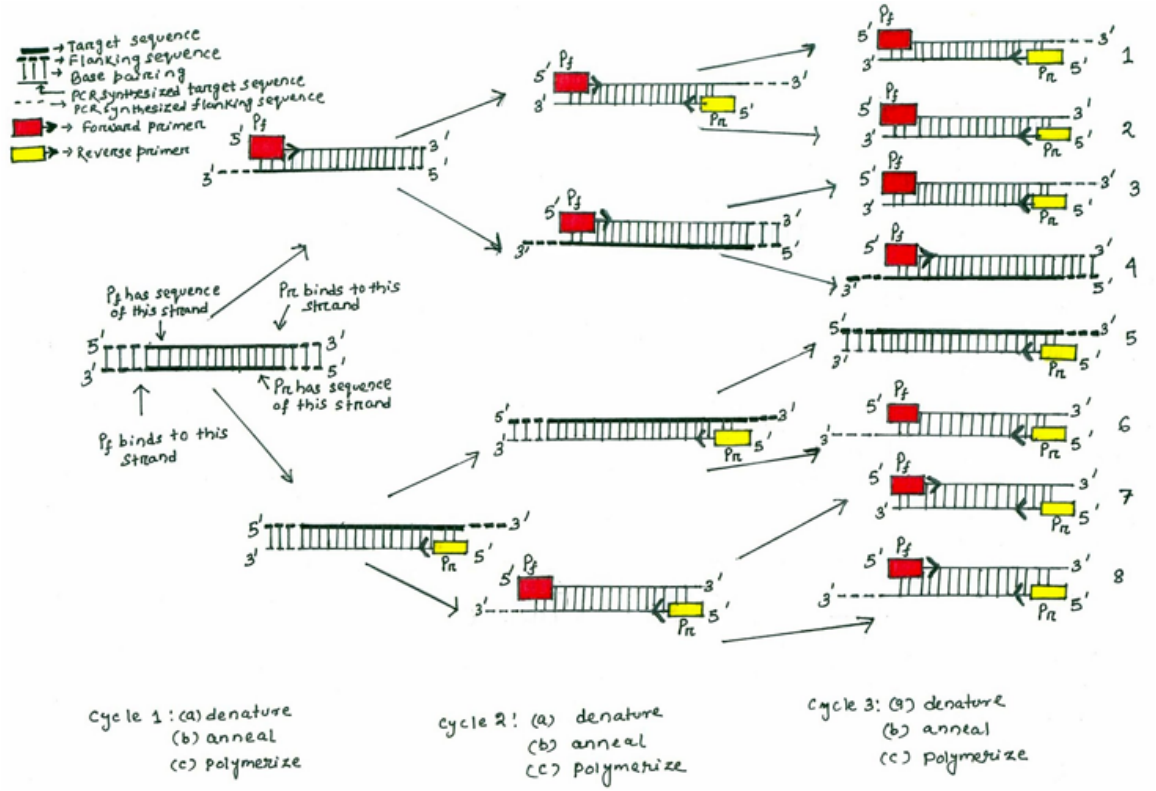

Figure 3. Reproduced from http://www.discoverbiotech.com/wiki/-/wiki/Main/Polymerase+Chain+Reaction showing the first 3 cycles of a PCR reaction. Cycling the temperature between 95°C for denaturation, 50-65°C for annealing and 72°C generates two copies for each one in the previous cycle.

1.5.2.1 Real-time PCR

Real-time PCR combines a PCR with a fluorescent reporter to monitor the process of amplification. Such an approach removes the need to analyse products on a gel and provides more accurate quantification. There are two common methods of detection of PCR products, the first method uses an intercalating dye such as ethidium bromide which binds non-specifically to double stranded DNA meaning the fluorescence is proportional to the mass of DNA in the reaction. The second method uses a probe with a

fluorescent reporter at the 5' end and a quencher at the 3' end. This binds within the target and is cleaved by the exonuclease activity of the polymerase during extension. Once it is no longer in close proximity to the quencher, the dye fluoresces. The advantage of this system is that non-specific products such as primer dimer do not contribute to the fluorescence. Multiple dyes with different emission wavelengths can be multiplexed in the same reaction as long as the wavelength is supported by the qPCR instrument.

1.5.2.2 The reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR a variant of PCR which enables amplification from an RNA template by incorporating a reverse transcription step at the start to generate cDNA. Standard PCR is then used to amplify a target from the cDNA. Reverse transcription in the 3' to 5' direction can be primed either using a specific primer such as oligo(dT) for poly(A) tailed mRNA or a random hexamer primer. If using a specific primer, reverse transcription and PCR can be performed in a single reaction known as one-step RT-PCR, or RT-qPCR if quantitative. This method is common in diagnostic assays for viral infections as it involves fewer pipetting steps which reduces the chance of contamination. Using the fluorescence data collected during the run software will determine the cycle threshold (Ct) value, which is the cycle that the fluorescence increased above baseline. This value is inversely related to the copy number of the target in the starting sample.

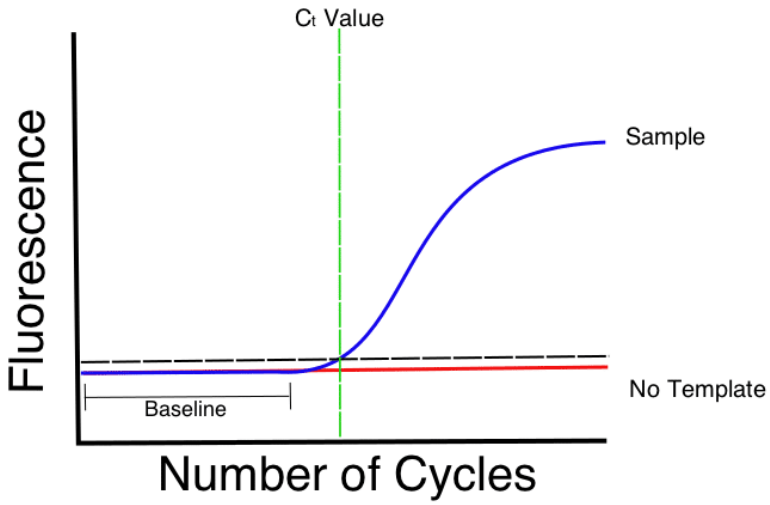

Figure 4. Reproduced from https://bitesizebio.com/24581/what-is-a-ct-value/ showing a simulated amplification qPCR curve, Ct value is the cycle where the fluorescence curve intersects the threshold based on the background fluorescence. Amplification curves are sigmoidal as they are exponential in early cycles then plateau as the reagents are depleted.

1.5.2.3 Multiplex PCR

Multiplex PCR is the process of amplifying multiple targets in a single reaction by the inclusion of primers for more than one target. This could be in order to multiplex targets in the same qPCR assay, each with its own detection channel or for generating a pool of amplicons for sequencing with next-generation sequencing. Mixed pools of amplicons must be sequenced because only products of different lengths can be differentiated on a gel. This is also true of single genes amplified from mixed populations such as with barcode sequencing like the bacterial small ribosomal subunit 16S or mitochondrial CO1.

1.5.3 Pulsed-Field Gel Electrophoresis

Pulsed-Field Gel Electrophoresis (PFGE) is a typing method in which the restriction digest patterns of different bacterial strains are compared. Genomic DNA is digested using a restriction endonuclease and the products are run by gel electrophoresis. It is not easy to separate molecules above 40 kb with a constant electrical field. In order to resolve fragments larger than this a pulsed-field system is required. Fragments up to several megabases in size can be resolved by pulsed-field by switching the direction of the electrical field which means molecules can zigzag through the gel. This works because it takes longer for large molecules to reorient in a fluctuating field which means they travel slower for a given set of switching conditions[17].

1.6 DNA sequencing

DNA sequencing by the incorporation of chain-terminating di-deoxynucleotide triphosphates (ddNTP's) was invented by Fred Sanger in 1977[18]. He found that by including a certain ratio of each ddNTP in four separate reactions, then performing a template extension by DNA polymerase he could read the sequence by gel electrophoresis of each reaction. This technology was used to sequence one the first complete genomes, of bacteriophage λ, earning Sanger his second Nobel prize.

1.6.1 Multilocus sequencing typing (MLST)

The development of the polymerase-chain reaction (PCR) and Sanger sequencing led to the adoption of sequence-based typing methods. One of the most popular techniques is MLST in which sequence variation is determined in multiple (usually seven or eight) 'house-keeping' genes. 450-500 bp fragments are amplified by PCR and sequenced[19]. The sequences at these loci are compared against those already in one of two databases (mlst.net/ and pubmlst.org/) to see if the allele has already been assigned a number. If the allele is novel a new number will be assigned centrally after verification. The set of alleles numbers is known as the allelic profile or the sequence type which are also curated in the database. MLST involves sequencing PCR products which makes it more reliable than PFGE which relies on laborious agarose plug extractions taking several days. It has represented a 'gold standard' in sequence typing for many years and a well-designed scheme can provide good clustering however for genetically monomorphic species such as *M. tuberculosis* it can lack discriminatory power[20]. Today sequence types are still widely used however due to the continued decline in the cost of next-generation sequencing (see 1.6.3) alleles are usually extracted from data generated using shotgun sequencing (1.6.2) often referred to as *in silico* sequence typing[21].

1.6.2 Genome sequencing projects

With the introduction of automated Sanger sequencing instruments in 1987, the sequencing of larger genomes became conceivable. Consortia were set up to sequence bacterial model species such as *Escherichia coli* and *Bacillus subtilis*. The Human Genome Project (HGP) was set up in 1990 with the even bigger ambition of sequencing the human genome. They planned to do this by the top-down method, firstly using

traditional linkage maps, followed by cloning of ordered large fragments of the genome into bacterial artificial chromosomes (BACs). These BACs were then fragmented and then assembled individually, before being combined into a larger whole-genome assembly. In 1995 Craig Venter stunned the world with the announcement that his team had sequenced the first bacterial genome, *Haemophilus influenzae* in just twelve months[22] using a pure whole-genome shotgun technique that bypassed the need to produce physical maps or BACs. A few months later they published the genome of *Mycoplasma genitalium*[23] the smallest known genome of any free-living organism. More complex genomes followed with *Saccharomyces cerevisiae* (12 Mb) in 1996 and *Caenorhabditis elegans* (100 Mb) in 1998 from other groups using the direct shotgun method. At the time it was not thought that the whole-genome shotgun method alone would be sufficient for large complex genomes such as human. However, by combining it with mate-pair and state of the art assembly software the private Celera corporation assembled *Drosophila melanogaster* (175 Mb) in 2000 and human (3.12 Gb) in 2001. Both the HGP and Celera simultaneously published manuscripts outlining drafts of the human genome assembly in Nature and Science respectively[24, 25]. The scale and complexity of these genome sequencing projects led to the rapid growth of very large specialist industrial-scale genome sequencing centres such as the Sanger Institute.

1.6.3 The second revolution: next-generation sequencing

The introduction of next-generation sequencing (NGS) using massively parallel sequencing dramatically reduced the cost of sequencing whole genomes. The launch of the 454 GS20 in 2005[26] was swiftly followed by the Solexa Genome Anayzer in

2006[27], both using a sequencing-by-synthesis (SBS) approach i.e. detecting single base incorporations by DNA polymerase into a growing population of clonal DNA clusters. The chemistry system used by 454 uses emulsion PCR to amplify library molecules on beads inside droplet reaction chambers. The sequence is determined by a microfluidic version of the existing pyrosequencing method in which a cocktail of three enzymes generate a detectable light signal: DNA polymerase which incorporates a dNTP releasing pyrophosphate, ATP sulfurylase which converts pyrophosphate to ATP and firefly luciferase which consumes ATP to produce light. In this system the intensity of light is approximately proportional to the number of bases incorporated at a given step. The Solexa chemistry by contrast uses a 'reversible terminator' chemistry with each base having a different fluorescent label. Bases are incorporated one at a time, but can be unblocked chemically to permit the next cycle of chemistry to proceed. A light source and a camera is used to read the most recently incorporated nucleotide's fluorescent tag. Solexa was bought by Illumina in 2007 who have developed the SBS chemistry to increase read lengths to up to 300 bp and run throughput to up to 6 terabases ($6x10^{12}$ bases). The scalability proved decisive and in 2016 the 454 platform was withdrawn from sale due to uncompetitive running costs.

1.6.3.1 Bench top instruments

The Illumina MiSeq, released in the autumn of 2011, was one of a new breed of 'benchtop' sequencers and has become heavily used for viral and bacterial whole-genome sequencing[28]. Users are able to generate sufficient data to sequence up to 96 bacterial genomes in 2-3 days for around $100 per sample. This meant universities and

public health laboratories, by running their own instrument instead of utilising academic genome facilities, could dramatically reduce the time taken to generate results. This facilitated the adoption of sequencing in cost restricted environments such as the UK's National Health Service for performing bacterial typing, antibiotic susceptibility prediction and surveillance sequencing in a single assay[29].

1.6.4 The Third Revolution: Single-Molecule Sequencing

1.6.4.1 PacBio SMRT sequencing

The Sequel and RSII instruments sold by Pacific Biosystems (PacBio) use Single Molecule Real-Time sequencing (SMRT) sequencing to detect incorporation of fluorescently labelled bases by a polymerase in real-time. Sequencing reactions take place in zero mode waveguides (ZMWs) each too narrow for light to propagate into. The ZMWs each house an immobilised template-polymerase complex in the illuminated region at the bottom. Phospholinked nucleotides are present in the reaction chamber but only those being incorporated by the polymerase are in the excitation zone long enough to be detected, observed as flashes of light. As the fluorophore on the phosphate chain is cleaved off by the polymerase during incorporation and the fluorophore diffuses away. A movie is recorded then later analysed to determine the sequence of each temple. Although a sequencing-by-synthesis approach, modifications such as methylation can be inferred from their effect on the enzyme kinetics, relying on the observation that modified bases typically are slower to incorporate than unmodified bases[30].

1.6.4.2 History of Nanopore Sequencing

The concept for using nanopores to sequence DNA molecules directly was first devised by David Deamer in 1989[31], yet it has taken 25 years to overcome the many technical hurdles to develop the first commercial system. The first experimental evidence that the system could be used to sequence nucleic acids was produced in 1996 when Kasianowicz et al., who had been working closely with Deamer, detected translocations of RNA homopolymers through an α-hemolysin pore[32]. As the technical details were fleshed out it became apparent that the translocation speed through he sensing region was on the order of 1-10 μs per nucleotide, even at low bias voltages. A way of ratcheting the DNA through the pore at slower speed was needed. After some mixed results using different classes of DNA polymerase, Mark Akeson discovered that phi29 polymerase could be coupled to protein nanopores to ratchet DNA through the pore at 2.5-40 nucleotides per second at single nucleotide resolution[33]. Crucially the enzyme could also be nanopore activated i.e. did not polymerise in the *cis* compartment when a negative bias voltage was applied.

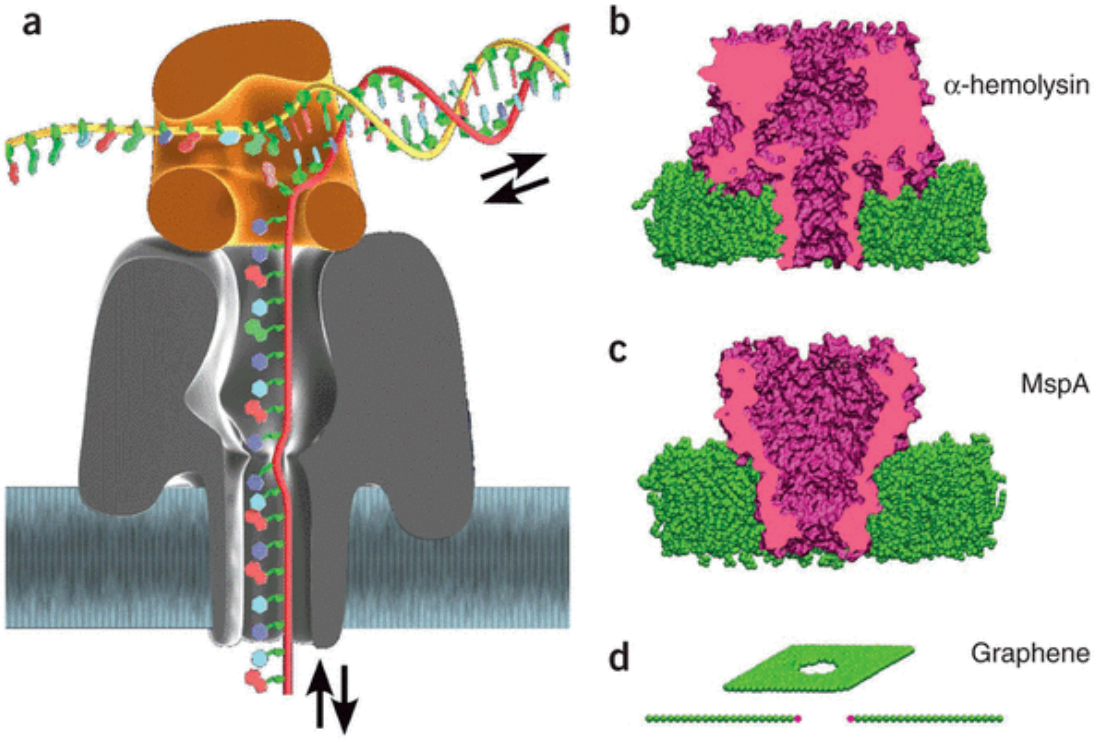

Figure 5. Reproduced from[34]. (a) ssDNA translocating through the nanopore with phi29 DNA polymerase acting as a ratchet. Panels (b-d) show different types of pores that have been used to detect DNA.

1.6.4.3 Principles of nanopore sequencing

The key feature of a nanopore sequencing system is the nanoscale pore which is inserted into a membrane, traditionally a lipid bilayer. Either side of the membrane, the *cis* and *trans* compartments, are filled with ionic solutions. When a bias voltage is applied across the membrane, ions flowing through the pore produce an electrical current which can be detected using a sensitive ammeter. Negatively charged DNA molecules, when added to the *cis* compartment, are driven electrophoretically through

the pore. The disruption to the ionic current by the mass and electrical field of the bases in the pore cause a shift in the measured current. High-frequency sampling of the current as a DNA molecule is ratcheted through the pore generates an electrical current trace measured in pico-amps. The DNA sequence can be deduced by comparing the current trace to ones trained on known sequences. The ratchet enzyme slows down the translocation orders of magnitude allowing each base to remain in the pore long enough to detect it.

1.6.4.4 Commercialisation of nanopore sequencing

In June 2014, the MinION developed by Oxford Nanopore Technologies (ONT) became the first nanopore sequencing device to market. MinION was brought to market with a 'Gillette' cost model; low instrument price and profits generated on sales of disposable flowcells. It represented a significant departure from all instruments before it being only the size of a USB dongle and drawing power from a laptop computer. The flowcell itself contains up to 2048 individual nanopores with a 4:1 multiplexer allowing data collection from 512 simultaneously using the MinKNOW control software, also developed by the company. The system was launched using an undisclosed protein pore. In March 2016, ONT announced that future products would utilise mutants of CsgG pore a lipoprotein from *E. coli* now known as 'R9' (https://nanoporetech.com/events/no-thanks-ive-already-got-one). The system employed a DNA helicase to act as the ratcheting 'motor' protein. The helicase is bound to the sequencing adapter but held *in situ* on a stretch of the adapter until activated by the electrophoretic force as it enters the pore. Another important innovation was the

inclusion of a cholesterol group which tethers the library onto the membrane. This produced a thousand times increase in sensitivity with respect to bulk phase sampling. The current traces (known as 'raw signal') are written into HDF5 format 'FAST5' files by the data collection software MinKNOW[35]. Basecalling is performed directly from raw using software called Albacore. Albacore is a neural network type basecaller using a model trained on E. coli, S. cerevisiae and human data. Homopolymers translocating do not result in current shift, but a type of neural network known as a transducer network is used which is capable of estimating the length of a homopolymer from the dwell time improving the accuracy of these basecalls (https://github.com/nanoporetech/scrappie).

1.6.5 Sequencing library preparation methods

Sequencing libraries for next-generation sequencing consist of a pool of fragments which could contain anything from one to trillions of unique molecules. It is in this regard where next-generation technologies fundamentally differ from Sanger sequencing and where the term massively-parallel sequencing originates from. Instead of generating copies of the template using cloning or PCR, amplification takes place on beads or attached to a surface forming a 'colony'. This enables sequencing reactions to take place as a two-dimensional array of features facilitating detection. Generating libraries usually consists of taking some fragments of DNA or RNA and adding adapters onto the ends making them compatible with the sequencing chemistry.

1.6.5.1 PCR and PCR-free libraries

Most types of NGS library preparation involve ligation of adapters for PCR amplification or sequencing. PCR amplified libraries suffer from GC bias, where GC rich or poor fragments are underrepresented in the library as a result of inefficient amplification. PCR-free libraries can be made with higher input however this does not fully alleviate the issue as 454 and Illumina sequencing require amplification for colony generation. By contrast, single molecule sequencing technologies do not require amplification and therefore should be immune to GC bias. Paradoxically, despite sensing single molecules they require extremely high amounts of input DNA. A typical Illumina or Oxford Nanopore PCR-free library requires 1 μg input material. The use of gel-based size-selection increases DNA input further due to sample losses. For PCR libraries Illumina Nextera XT libraries can be generated from as little as 1 ng using 12 cycles of PCR. Generating PCR-free libraries is not practical for all sample types e.g. low biomass samples so library preparation methods for a variety of inputs are required.

1.6.5.2 Mechanical fragmentation

Genomic DNA (gDNA) is fragmented when a specific fragment size distribution is desired for a sequencing library. That may be a platform-specific decision such as matching the insert to the read-length in Illumina sequencing (typically 500-1000 bp) but can also occur when handling especially high-molecular-weight DNA and is usually unwanted. For Illumina sequencing in which a desirable range might be 100-1500 bp, fragmentation is often performed using Covaris focused-ultrasonication. For fragments

longer than 5000 bp hydrodynamic shearing using a Covaris g-TUBE or needle shearing gives more consistent results. In this method DNA molecules experience shearing forces as they pass through a narrow opening resulting in fragmentation.

1.6.5.3 Ligation libraries

In order to ligation adapters to random fragments they must first be end-repaired and dA-tailed. This is performed using a combination two enzymes, T4 DNA polymerase and Klenow fragment which remove 3' overhangs and fill in 5' overhangs. dA-tailing is used to avoid the formation of adapter concatemers during ligation. This adds to the time required to generate a ligation library. For systems that require colony amplification different adapters are required on each end of the library construct e.g. Illumina paired-end sequencing. Using two adapters (A and B) the possible ligation products also include A-insert-A and B-insert-B which will not amplify which results in low efficiency. The solution to this was the 'Y'-adapter introduced by Solexa which uses a complementary region with two non-complementary regions needed for cluster amplification. During the first PCR cycle a single short primer generates two asymmetric products which are then amplified by a tailed long primer and the short primer. Oxford Nanopore libraries cannot be generated by PCR as the adapters contain modifications and the motor protein. If PCR amplification is used then adapters will be added by a separate adapter ligation step after.

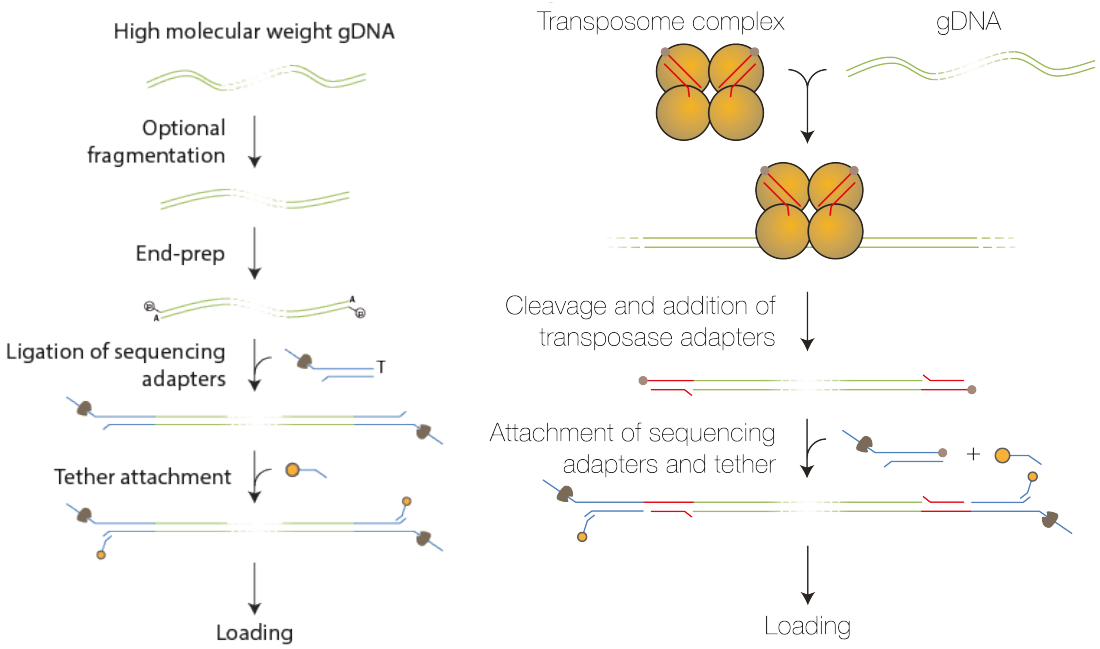

Figure 6. Reproduced from https://community.nanoporetech.com. This figure illustrates three common methods for library preparation for Oxford Nanopore sequencing by end-preparation and adapter ligation (left) or by tagmentation and adapter ligation (right).

1.6.4.4 Transposase libraries

An alternative to mechanical fragmentation is enzymatic fragmentation. This could be done using endonucleases either specific or non-specific or more desirably using a transposase enzyme. Transposases are the enzymes that facilitate the movement of transposons around the genome. They are now widely used in next-generation sequencing library preparation because they can perform a dual function of fragmenting and adapting genomic DNA. Transposases are complexed with double stranded adapters by incubating in the absence of cofactor Mg^{2+} forming a transpososome, these adapters will eventually be attached to the ends of the DNA at the cut sites in a process known as tagmentation. The length of fragments generated by tagmentation will be dependent on

the starting fragment size, the mass of DNA and the molarity of transposomes. By controlling the molarity of transpososomes you can control the molarity of tagmented fragments and increasing the mass of input DNA will increase the fragment size. These enzymes are used in both the popular Nextera XT kit (Illumina) utilising a Tn5 mutant and the Rapid sequencing kit (Oxford Nanopore Technologies) utilising MuA transposase. By combining fragmentation and adapter attachment these methods are among the fastest library preparation methods in use and are also compatible with PCR and barcoding. They are not suitable for amplicon sequencing as they cut in the middle of fragments resulting in lower coverage of the ends of the amplicon with respect to the middle.

1.6.5.5 Sample barcoding

Molecular barcoding is used to multiplex many samples into the same flowcell or lane, this way the barcode can be sequenced in order to separate reads later in a process known as demultiplexing. Barcodes are short sequences introduced during library preparation which allow multiple samples to be pooled in a library. They are usually 6 or 8 bases long in Illumina sequencing or 24 bases for Oxford Nanopore due to the lower basecalling accuracy. They can be introduced either during PCR, by a transposase or by ligating double stranded oligonucleotides to fragments in PCR-free libraries. Dual indexing strategies using different barcodes on each end of the molecule allow for large number of barcode combinations i.e. 96 combinations are achieved using 8 × 12 barcode sequences. Multiplexed sequencing can be used to reduce the cost per sample when the full output of a flowcell or lanes excessive for a single sample.

1.7 Comparative genomics

The success of Sanger shotgun sequencing and then next-generation sequencing launched the field of comparative microbial genomics. Analysis of the genome of *Mycobacterium leprae*, a species adapted to an isolated niche, found it had undergone extensive genome reduction compared with *Mycobacterium tuberculsosis*. This was characterised by accumulation of pseudogenes or the complete deletion of genes no longer needed for survival[36]. By 1999 there were two genomes available for the same species[37], *Helicobacter pylori*. The genomes were found to be quite similar in structure and gene order. A small percentage of the genes were unique to one strain or the other, with half of these genes clustered in a hypervariable region of lower %GC suggesting they could have been acquired by horizontal gene transfer.

1.7.1 Horizontal gene transfer

Horizontal gene transfer is the transfer of genetic material from one organism to another. In prokaryotes it can occur in three ways; transformation, transduction or conjugation[38]. Transformation is a natural process by which competent cells bind, take up and recombine exogenous DNA into its chromosome. Integrated DNA is usually derived from a closely related organism so occurs by homologous recombination. Transduction is the process by which DNA is transferred from one cell to another via a viral vector. This occurs when small pieces of bacterial DNA, either adjacent to the phage insertion site or from somewhere else in the bacterial genome are packaged into the phage genome. In what is known as ‘generalised transduction’ a

stretch of bacterial DNA is packaged into the viral envelope by accident. If the phage infects another bacterial cell the DNA can integrate into the hosts genome by homologous recombination. In the alternative method ‘specialised transduction’ bacterial genes with close proximity to the prophage are included in the excised DNA. This is then packaged into a new virus, which after infecting another cell may be integrated into its genome. Conjugation or ‘bacterial sex’ is the exchange of a genetic material, either a plasmid or transposon via direct cell-to- cell contact.

1.7.2 Recombination

Recombination in prokaryotes can be homologous or non-homologous. Homologous recombination occurs between closely related organisms due to high sequence similarity, with the frequency of homologous recombination events falls exponentially with decreasing sequence identity. Non-homologous recombination can occur between organisms with no sequence homology as it utilises a double strand break repair system. As non-homologous recombination events may reduce the likelihood of homologous recombination they may be serve as speciation ‘seeds’ which lead to diversification between strains[39].

1.7.3 Plasmids

Plasmids are extrachromosomal replicons (replicative unit), that are inherited by daughter cells during cell division and are also capable of horizontal transfer into other species or genera. They typically vary in size from a few kilobases to hundreds of

kilobases and are found throughout the bacterial and archaeal kingdoms. Plasmids may carry genes ('cargo') that may confer a selective advantage such as resistance to a particular antibiotic. Some may not have any useful function yet are maintained by 'addiction systems' such as toxin-antitoxin system in which a plasmid encodes both a toxin and an antitoxin, meaning the bacterium will be killed by the toxin if the plasmid if lost.

1.7.4 Integrative conjugative elements

Integrative conjugative elements (ICE) are similar to conjugative plasmids however they lack an origin of replication and the genes required to make the conjugation machinery, they have to therefore integrate into a chromosome or plasmid in order to persist. The advantage of this is they can integrate into a broader range of hosts, as they do not reply on conjugation machinery being compatible with the host

1.8 Epidemiology

1.8.1 History

Epidemiology is the study of patterns of disease within a population. John Snow, known as the father of modern epidemiology, famously investigated a cholera outbreak in the Soho district of London in 1854[40]. By talking to local residents and creating a map of cholera cases he was able to identify a water pump as the source of the outbreak. Removing the water pump's handle ended the outbreak. Both Pasteur and Koch also

performed important epidemiological research. Pasteur travelled to the south of France to investigate a disease of silkworms called *pébrine* in research spanning five years. Koch travelled to Egypt and India searching for the cholera microbe and made some important observations about the importance of water in public health.

1.8.2 Genomic epidemiology

Genomic epidemiology is the application of genomic sequencing to the practice of disease epidemiology. Whole-genome sequencing permits phylogenetic reconstruction to be performed, providing an additional source of information about the relatedness of cases. This information has been used to determine the source of the 7th global cholera pandemic[41]. Originating from the Bay of Bengal analysis shows that it emerged in three overlapping waves. The emergence of cholera, caused by *Vibrio cholerae*, in Haiti after the 2010 earthquake resulted in 4900 deaths. Phylogenetic reconstruction showed isolates from the Haiti outbreak were most closely related to South Asian strains suggesting that Cholera had been imported to the island. After international pressure a UN investigation found those who had drunk contaminated water from the Artibonite river had become sickened. This added weight to suspicions that a UN peacekeeping force from Nepal were the most likely source of the outbreak as they had a camp on one of the river's tributaries[42].

1.9 Bioinfomatics methods

1.9.1 Primary analysis

Tools for primary analysis of sequence data generally fall into two categories; alignment (reference-based) and *de novo* assembly (reference-free). An underlying feature of both of these techniques is decomposition of sequences into *k*-mers, all possible substrings of length *k* contained in a string. Comparison of fixed length strings is extremely fast. Using shorter *k*-mers increases the likelihood of finding exact matches even in the presence of sequencing errors or biological variation. In alignment, *k*-mers are used to find alignment seeds whereas in de Brujin graph assembly the graphs are built from *k*-mers. Modern taxonomic classifiers use *k*-mers for fast database lookup so they are fundamental to bioinformatics analysis.

1.9.2 Alignment

1.9.2.1 Short read aligners

Pairwise alignment is the comparison of two sequences, typically with DNA, RNA or amino acid alphabets. The first alignment algorithm and foundation for subsequent techniques was the Needleman-Wunsch algorithm[43]. This is able to find the best scoring global alignment of two whole sequences. A development of this, the Smith-Waterman algorithm was able to find the best local alignment i.e. a subsequence of the whole sequence[44]. Aligners designed for short-reads such as bwa[45], use the Burrows-Wheeler transform (BWT) of the reference with backward search to find

alignments. This technique is very efficient at aligning short reads against a large reference, such as human and allows mismatches and gapped alignments.

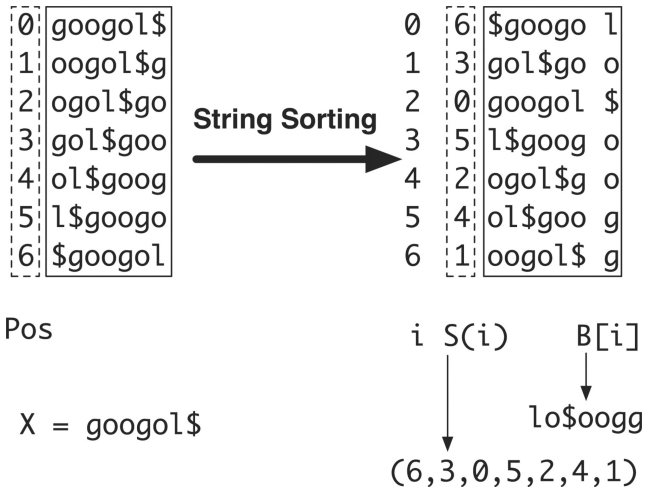

Figure 7. Reproduced from[45]. A Burrows-Wheeler transform of the reference is generated by taking the input string 'google' and generating all possible rotations, before sorting them lexicographically and taking the last column as the output.

1.9.2.2 Querying databases

As of October 2017 there are over 200M sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/statistics/) and querying them quickly and accurately is a big challenge. It would be too slow to find the optimal match using Smith-Waterman so algorithms such as BLAST[46] use a hash table index to hold the positions of each *k*-mer (default *k*=11) subsequence in the query. It then searches the database for exact matches or 'seeds' and extends the highest scoring ones based on a heuristic method which is less accurate than Smith-Waterman but much faster. An important feature of BLAST results is the E-value, the statistical significance of the alignment, the lower the E-value the less likely the match is to have occurred by chance.

1.9.2.3 Long-read aligners

Several aligners have been developed for either PacBio or nanopore long reads. They have typically been slower than their short-read counterparts. The exception to this is minimap2[47] which has designed for aligning noisy long-reads to large references. The algorithm uses query minimizers (reduced *k*-mer representations) as seeds and finds matches in reference minimizers. Smith-Waterman is used to extend co-linear seeds, called chains to generate the alignment. This approach is extremely efficient for long-reads and achieves higher performance in terms of aligned bases per second than short-read aligners. This is because long-reads are more likely to align uniquely, one of the main bottle necks in short-read alignment.

1.9.3 Genome assembly

Genome assembly is process of identifying overlaps in reads in order to build longer sequences. An early assembly method was Overlap-Layout-Consensus devised by Gene Myers, as utilised by the Celera Assembler. Long reads have many advantages over short reads for genome assembly such as their ability to span many classes of repeats. Assemblers for long-reads however need to be able to handle noisy reads, as both single-molecule platforms PacBio and Oxford Nanopore raw reads with an error rate of 5-20%. Much of this error is random so can be averaged out using increased coverage resulting in more accurate consensus sequences. This however requires an all-against-all alignment step which is computationally expensive. In early versions of the PacBio assembly pipeline HGAP[48], the overlapping step accounted for 95% of the run

time[49]. Adam Phillippy used a probabilistic approach called MinHASH which was originally developed to determine the similarity of web pages to tackle the problem. The algorithm known as MHAP and associated assembler Canu, which is based on the Celera assembler achieved 10x performance improvement on smaller genomes and 100x on larger genomes compared to previous methods.

1.9.3.1 Assembly polishing

Long-read assemblers such as Canu[50] will produce contigs with a similar error rate to the corrected reads used for the overlap-layout consensus. An additional step known as polishing is needed to correct short insertion, deletion and substitution errors in the assembly. This is done using a by aligning the original reads back to the contigs and calculating a consensus sequence. For PacBio assembly, Quiver[48] or Arrow is used which predicts the most likely consensus base using additional quality information from the raw reads.

1.9.3.2 Error correction using Illumina data

Provided sufficient coverage, long read-only assemblies will be more contiguous than either short-read only or hybrid assemblies. This is because hybrid assemblers typically use long reads for scaffolding (joining) contigs rather than for building them. After assembly, polishing long-read assemblies can be very accurate, with PacBio able to generate Q50 bacterial genomes (99.999% accurate). Despite this it is often still desirable to generate low coverage Illumina data for correcting the residual errors,

bringing the assembly up to reference quality. The *E. coli* K-12 genome is around 4.6 Mb in size meaning a Q50 genome could still contain 45 errors (PacBio) or 1300 errors (Oxford Nanopore). In both platforms, the majority of the errors will be indels associated with homopolymeric tracts. These can be corrected by incorporating Illumina short-reads which have a mean raw read accuracy of Q20 but rarely contain indel errors. Pilon[51] is a tool able to generate a corrected consensus given a BAM file of Illumina short-reads aligned to a polished long-read assembly.

1.9.4 Nanopolish

Because Oxford Nanopore data differs significantly from other sequencing technologies a separate tool, Nanopolish is needed to calculate consensus, variant calling and methylation detection using the underlying signal. The first stage of the algorithm is to detect events, a type of partitioning used to represent the electrical current level of a single *k*-mer in the pore. The consensus algorithm iteratively proposes alternate consensus sequences and selects the one that maximises the probability of observing the events as calculated by a profile HMM. The Nanopolish HMM in consensus mode uses proposed consensus sequence as the backbone of the HMM with additional states and transitions to handle missed and split events. The method can produce a consensus sequence *for E.* coli that is 99.97% accurate[52] with the residual error mainly homopolymer associated SNPs or indels, a systematic error mode in nanopore sequencing data.

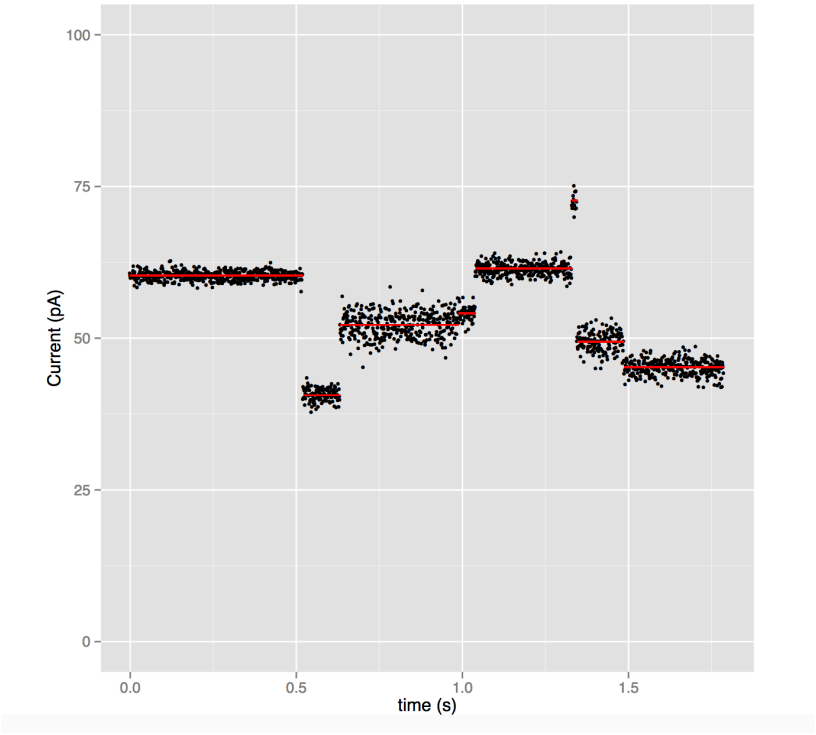

Figure 8. Reproduced from http://simpsonlab.github.io/2015/04/08/eventalign/ this plot shows raw samples (black) and events (red) for simulated data. These can vary dramatically in length due to the imperfect ratcheting causing the event detector to make errors.

1.9.4.1 Variant calling from nanopore data

In order to perform variant calling, Nanopolish first infers the event to reference mapping for each base from the alignment of each read to the reference. Following this, candidate SNPs are detected by finding positions in the alignment with an alternate base frequency above a defined threshold (typically 20%). It then clusters these into haplotype groups before testing all possible haplotype combinations before calculating the likelihood of each using the Nanopolish HMM. Calling haplotypes rather than

individual SNPs improves the sensitivity of Nanopolish when calling large number of variants or using a divergent reference.

1.9.5 Variant calling in Illumina data

Variant calling using Illumina short-reads is a relatively simple procedure due to the high accuracy, although the shorter reads may predispose aligners to make false mappings. Reads are first mapped either against a closely related reference genome or an assembly using an aligner such as BWA[45]. Aligning to a closely related assembly is preferred because mapping quality and numbers increase. A goal is to be able to perform 'forensic-quality' SNP calling in order to distinguish strains which differ by as little as a single mutation. When variant calling groups of distantly related strains or strains without a closely related reference then the choice of reference genome is less important, but variant calls are limited to the conserved set of 'core' genomic regions, that is the ones shared by all strains. A BAM file for each sample is given to a variant caller such as VarScan[53] which iterates over the alignment outputting calls to a VCF file. Typically variant calls are filtered to improve reliability of calls using fixed criteria based on coverage depth, variant frequency and base or mapping quality.

1.8.5.1 Functional annotation of variants

To predict the effect of variants e.g. SNPs and indels, functional annotation is performed. The software SnpEff[54] is able to predict effects based on whether a mutation is; synonymous i.e. results in a codon that produces the same amino acid; non-

synonymous i.e. results in a codon that produces a different amino acid; stop gained i.e. variant causes a new stop codon or frame shift i.e. an indel causes a fame shift. These can be assigned a severity as a synonymous change will have no phenotypic effect whereas a frame shift or new stop codon is likely to result in a loss of function of that protein. Using a VCF file input SnpEff will add predicted functional annotations to the information column in the VCF file.

1.9.6 Bacterial annotation

Identifying the various features in a bacterial genome is a process known as annotation. Features annotated may include coding sequences, ribosomal RNA genes and transfer RNA genes. Prokka[55] is a convenient pipeline which takes an input FASTA file and uses external tools to identify these features and combines the output into database compliant standard format. Protein coding sequence detection is performed using Prodigal[56] to identify coordinates of putative genes by identifying start and stop codons. These candidate genes are then searched against databases at the protein level to find an annotation to transfer. It does this in a hierarchical manner from most to least trustworthy, using the databases UniProt (most), RefSeq and Pfam (least). If no annotation is found gene will be labelled as hypothetical proteins.

1.9.7 Tree building

1.9.7.1 Tree building from variant calls

Tree building software requires an input file of aligned sequences to generate a tree. This alignment could be a gene, core genome or just variant sites in a genome depending on the experiment. For basic phylogenetic inference, an approximate maximum likelihood tree such as that produced by FastTree is usually sufficient. FastTree starts with a topology produced by neighbour joining[57], a fast algorithm that uses a distance matrix to iteratively cluster sequences together. It then refines this topology using heuristics to restrict the search and estimates the rate of evolution for each site. Confidence in any branches are represented by bootstrap values, repeating the reconstruction on a subset of the data provides a way to approximate the sampling distribution which is unknown. Trees stored in Newick format can be visualised using software such as FigTree.

1.9.7.2 Bayesian phylogenetic inference

Bayesian phylogenetic interference relies on Bayes' theorem to calculate the posterior probability given a tree and parameters. In general, the posterior probability of trees cannot be calculated directly as it involves high-dimensionality space of all possible parameter values over all possible trees. Software such as BEAST[58] uses Markov Chain Monte Carlo (MCMC) sampling to perform a random walk over the space. The number of times the algorithm visits a particular tree is proportional to the likelihood of that tree given the data. The chain length can be a million iterations long including a burn-in period which is discarded (10% by default). The algorithm is said to have converged or run for sufficient iterations when the trace of the posterior probability looks like a 'hairy caterpillar'. An important feature of BEAST is that is can use the

coalescent model[59] (Figure 9) to estimate demographic function e.g. exponential growth and evolutionary rate from the tip dates.

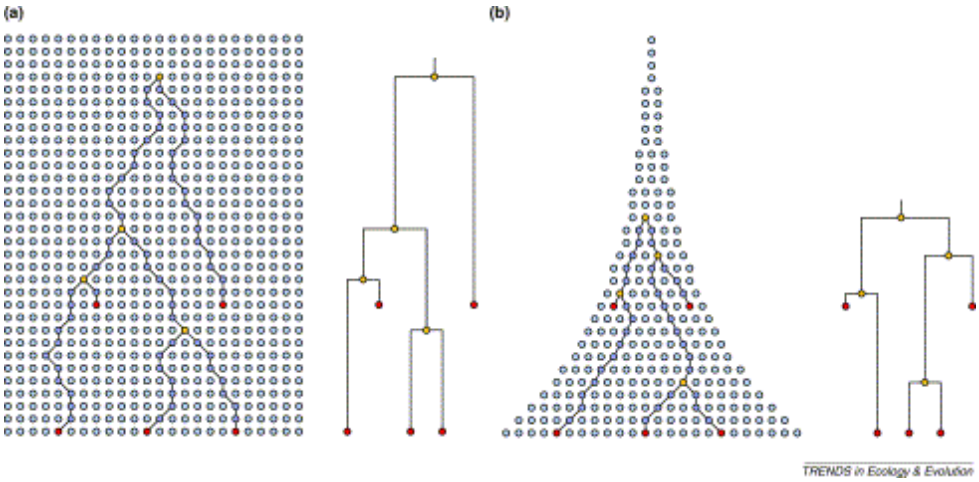

Figure 9. Figure reproduced from[60] illustrating the coalescent model. Figure shows the relationship of individuals samples from a constant population (a) and an exponentially growing population (b). As you travel back in time the probability of a coalescence event is inversely proportional to the population at a given time meaning the population size can be inferred from the pattern of coalescence and sampling events.

1.9.7.3 Phylogenetic placement

Phylogenetic placement is the process of placing a query sequence onto a reference guide tree without having to build a new tree each time. This is useful in situations where a) you need the result very quickly or b) have so many sequences that doing a full reconstruction is impractical. Pplacer[61] can place queries using either maximum-likelihood or Bayesian mode. In maximum-likelihood mode the software evaluates the maximum-likelihood values across all placement locations. In Bayesian mode the

software evaluates the posterior probability of the new sequence being on an edge given the reference tree topology and branch lengths. As the reference tree is fixed the posterior probability can be calculated directly without the need for the MCMC sampling used in BEAST. Query sequences are treated independently so placement can be parallelised across processors.

1.9.8 Taxonomic classification

1.9.8.1 BLAST based classification

BLAST can be used to classify sequences from metagenomic datasets by finding the best aligning in a database of sequences such as the NCBI non-redundant database (nr). Despite being able to handle large reference databases it still takes a substantial amount of time to process several million short reads generated by a single Illumina MiSeq run. MEGAN[62] uses BLAST results but improves accuracy with short reads by implementing a lowest common ancestor (LCA) assignment algorithm. This assigns the lowest node that has all the hits as descendants based on NCBI taxonomy, meaning species specific sequences are assigned near the leaves and conserved sequences are assigned near the root of the tree. You can also use MEGAN to visualise the composition of the community and generate trees and abundance statistics at different taxonomic levels.

1.9.8.2 Abundance estimation software

Abundance estimation is general name given to classification software which achieves higher performance by using a reduced database which has been selected to contain marker sequences that identify microbial clades at species level or higher taxonomic levels e.g. MetaPhlAn[63]. Due to the reduced size database they only classify a small proportion of the dataset resulting in a relative abundance of organisms in a sample rather than a classification of every read. They are so much faster than BLAST based approaches they can scale to terabases of short read data e.g MetaPhlAn was used to classify 17 million reads from the combined Human Microbiome Project (HMP)[64] and Metagenomics of the Human Intestinal Tract (MetaHIT)[65] datasets, demonstrating the first practical large-scale analysis of metagenomic datasets.

1.9.8.3 k-mer based classification

Kraken[66] uses an alternative approach for taxonomic assignment of reads from metagenomics samples. It maintains the LCA algorithm of MEGAN but uses exact *k*-mer matching instead of inexact alignment which allows it to be significantly faster even than abundance based methods. It uses a database of *k*-mers and the LCA of all organisms whose genomes contain that *k*-mer, which can be built for any user-specified sequences but is also supplied with a default database MiniKraken DB built using k=31 from 2,256 complete genomes downloaded from RefSeq. More recently the idea has been extended via the software Centrifuge[67] which incorporates an FM-index which is able to efficiently do exact matching of *k*-mers of any length rather than a fixed value of *k*. Starting at the end of the query Centrifuge finds exact matches of k=16 and increases *k* until it reaches a mismatch. Centrifuge will also output multiple

classifications per read, 5 by default. If there are greater than 5 high scoring assignments it takes the LCA of the largest group recursively, until the number of assignments to 5 or less.

1.10 Research aims

This work aims to further the field of genomic surveillance by introducing faster, cheaper and more portable methods for pathogen sequencing. Genomic surveillance using whole-genome sequencing has unparalleled resolution as a typing and epidemiological tool. Current short-read technologies require specialist facilities to perform sample preparation, sequencing and analysis, which can take weeks to perform. The introduction of nanopore sequencing in 2014 promised that it could be used to perform real-time, portable sequencing but it would require the development of sample preparation methods and analysis tools tailored to the low throughput and accuracy of the MinION. In this work we sought to develop and integrate new methods for sample preparation, sequencing and analysis to tackle viral outbreaks in the field where data could be made available immediately enabling more effective interventions to be made.

2. Seeking the source of Pseudomonas aeruginosa infections in a recently opened hospital: an observational study using whole-genome sequencing

2.1 Author contributions

NC, CC and **JQ** did sequencing. **JQ**, NC, CMT and NJL analysed the data. NJL, NC, **JQ**, MJP and BO wrote the paper. All authors commented on the manuscript draft.

2.2 Author contributions (additional detail)

JQ produced the global phylogeny, the phylogenetic analysis, the sequence typing and the temporal/spatial analysis. JQ performed the metagenomics sequencing, taxonomic classification and genotyping analysis. JQ drafted all figures and assisted in writing the manuscript.

2.3 Abstract

Pseudomonas aeruginosa is an important opportunistic pathogen and a significant cause of morbidity and mortality. Burns patients are particularly at risk of infection due to the breakdown of the skin barrier. In this study patients admitted to hospital with severe burns had their wounds and hospital environment including the water outlets screened

for *Pseudomonas aeruginosa*. Whole-genome sequencing of 141 isolates from the study revealed frequent recovery of a single lineage we call ‘Clade E’ which is ST395 a known water associated clone. Five patients became colonised during the study period and in two cases the isolates recovered from the wound were indistinguishable from isolates recovered from water outlets from the room they were treated in. Using a near-complete reference genome generated for this clade we performed forensic level SNP calling which revealed micro diversity between outlets demonstrated the power of surveillance sequencing of environmental isolates as a way source tracking Pseudomonas aeruginosa infections in a hospital environment. In one case a thermostatic mixer value was removed for remedial work and we performed metagenomic sequencing of the biofilm removed from the valve. Using a phylogenetic placement method we found the genotypes recovered clustered with isolates from the outlets in room 9 the location it had been removed from.

2.4 Published manuscript

BMJ Open

Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: an observational study using whole-genome sequencing

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▸ Prepublication history and additional material is available. To view please visit the journal (http://dx.doi.org/10.1136/bmjopen-2014-006278).

JQ and NC contributed equally.

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ABSTRACT

Objectives: *Pseudomonas aeruginosa* is a common nosocomial pathogen responsible for significant morbidity and mortality internationally. Patients may become colonised or infected with *P. aeruginosa* after exposure to contaminated sources within the hospital environment. The aim of this study was to determine whether whole-genome sequencing (WGS) can be used to determine the source in a cohort of burns patients at high risk of *P. aeruginosa* acquisition.

Study design: An observational prospective cohort study.

Setting: Burns care ward and critical care ward in the UK.

Participants: Patients with >7% total burns by surface area were recruited into the study.

Methods: All patients were screened for *P. aeruginosa* on admission and samples taken from their immediate environment, including water. Screening patients who subsequently developed a positive *P. aeruginosa* microbiology result were subject to enhanced environmental surveillance. All isolates of *P. aeruginosa* were genome sequenced. Sequence analysis looked at similarity and relatedness between isolates.

Results: WGS for 141 *P. aeruginosa* isolates were obtained from patients, hospital water and the ward environment. Phylogenetic analysis revealed eight distinct clades, with a single clade representing the majority of environmental isolates in the burns unit. Isolates from three patients had identical genotypes compared with water isolates from the same room. There was clear clustering of water isolates by room and outlet, allowing the source of acquisitions to be unambiguously identified. Whole-genome shotgun sequencing of biofilm DNA extracted from a thermostatic mixer valve revealed this was the source of a *P. aeruginosa* subpopulation previously detected in water. In the remaining two cases there was no clear link to the hospital environment.

Conclusions: This study reveals that WGS can be used for source tracking of *P. aeruginosa* in a hospital setting, and that acquisitions can be traced to a specific source within a hospital ward.

Strengths and limitations of this study

- We have demonstrated that whole-genome sequencing can be used for source tracking of *Pseudomonas aeruginosa* in a hospital setting.
- We show convincing evidence that transmission has occurred directly from water to patients, but other routes are as likely.
- The main limitation of the study was the sample size, which could be attributable to interventions being carried out during the study.
- Our study focused on a burns unit and critical care unit in a newly built hospital. Modes of *P. aeruginosa* transmission may be different in hospitals with different styles of plumbing and on other augmented care units.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium and an important opportunistic pathogen in the healthcare setting. *P. aeruginosa* particularly affects those with impaired host or mucosal immunity and has a broad range of presentations including respiratory infections in cystic fibrosis and mechanically ventilated patients, bloodstream infections in premature neonates and wounds in burns injuries. Nosocomial *P. aeruginosa* outbreaks are frequently reported and associated with water sources such as taps, showers, mixer valves and flow straighteners, sink traps and drains.[1–10] Other potential routes of transmission include cross-infection, for example, carriage on the hands of healthcare workers, and through contaminated medical equipment such as endoscopic devices.[3 5]

In the UK, the role of water in the transmission of *P. aeruginosa* in healthcare settings has become a matter of urgent concern in response to a recent high-profile outbreak

affecting a neonatal critical care unit in Belfast in 2012.[11] This source was eventually determined to be sink taps.[11–13] National guidance is now in place detailing enhanced procedures for routine water sampling on augmented care units, with directed interventions such as disinfection and replacement of high-risk plumbing parts required.[14]

Historical phenotypic typing methods for *P. aeruginosa* such as O-antigen serotyping have more recently been replaced by molecular typing methods such as pulsed-field gel electrophoresis (PFGE), variable number tandem repeat analysis, random amplification of polymorphic DNA and multilocus sequencing typing (MLST).[15] These methods have been used to investigate outbreaks of *P. aeruginosa* within hospitals.[4 16–18] However, such techniques have important limitations for source tracking of infections in hospitals as they sample limited numbers of sites in the genome which may result in false clustering of unrelated strains.[19] In the past 5 years, whole-genome sequencing (WGS) has started to be used to investigate outbreaks in hospitals. WGS is attractive because of its digital, sharable format and ultra-high resolution, which is able to discriminate two isolates differing by just a single mutation. WGS has been successfully used to determine likely transmission chains during outbreaks of *Staphylococcus aureus*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*.[19–21] Benchtop sequencing instruments now offer a cost-effective approach for bringing bacterial WGS to the clinical environment.[22]

In this study, we explore the utility of WGS to determine the likely sources of *P. aeruginosa* in an at-risk population of burns patients. In the UK and US burns patients receive shower cart hydrotherapy as a mainstay of burns treatment.[23–26] A previous hospital audit suggested that up to one-third of such patients became colonised with *P. aeruginosa*. We hypothesised that this high rate of acquisition may relate to transmission from hospital shower water during therapy. We therefore wished to understand the importance of transmission from water compared with alternative routes such as cross-infection and endogenous carriage.

MATERIALS AND METHODS

Hospital setting

An observational, prospective study design was employed in a burns centre serving approximately 13.7 million people across the Midlands region of England with 300 admissions annually. Opened in June 2010, the burns centre comprises a purpose built 15-bed ward with 11 side-rooms and 2 dual-bedded rooms. Patients requiring mechanical ventilation and organ support are usually treated in two self-contained burns cubicles located within the trauma critical care unit. Despite the observational nature of the study, sampling was carried out during implementation of interim national guidance on control of *P. aeruginosa* issued by the Department of Health. These guidelines were issued in draft form March 2012, and subsequently revised in March 2013. This meant that parallel water sampling and engineering interventions were being undertaken during the period of study. In addition, some enhanced infection prevention measures were also introduced in response to an outbreak of a multidrug-resistant *A. baumannii*.

Study design and patient selection

Patients admitted to the burns unit were eligible for the screening phase of the study if they had burns injuries covering greater than 7% total body surface area (TBSA). Patients were screened as soon as possible after admission after they had given written informed consent. When appropriate, legal consultee advice was sought for patients lacking capacity due to emergency treatment. On admission, recruited patients were screened for carriage of *P. aeruginosa* (wounds, urine and stool) using standard microbiology techniques. Samples were then taken as part of routine microbiology service during the patients stay. Environmental and water samples were taken after the patient was admitted to the burns centre. If during the period of stay *P. aeruginosa* was isolated from a patient sample the patient was recruited into the second phase of the study. In this phase, patients had wound swabs taken at each dressing change as well as twice-weekly urine samples. The patient's environment and water from outlets in their bed space were sampled weekly for the duration of their stay, and after discharge (post-cleaning). Termination of the study was planned after 30 screening patient admissions, or a year, whichever came soonest, after which 10 patients were expected to acquire *P. aeruginosa*. This prediction was based on a previous local audit which suggested about one-thirds of burns patients became colonised with *P. aeruginosa*.

Microbiological and molecular methods

P. aeruginosa isolates were obtained from wound swab, urine, stool, environmental and water samples. *P. aeruginosa* was isolated from wound swabs, urine and stool by inoculation onto cysteine lactose electrolyte deficient agar (CLED) and cetrimide agar and incubation for 24 h at 37°C. Stool samples were cultured overnight in a cetrimide enrichment broth before subculture onto CLED. Identification was confirmed by resistance to C-390 and the VITEK 2 GN identification card. Antibiotic sensitivity assays were performed using the VITEK 2 AST N-210 card (bioMérieux, Basingstoke, UK).

The patient's environment (shower head rosette, drain, shower chair or trolley, bedside table, patient chair, instruments in contact with the patient) was sampled over a 10 cm^2 area by a Polywipe sponge. The sponge was placed in tryptic soy broth incubated for 24 h at 37°C then subcultured onto CLED and cetrimide agar. During water sampling, water was taken from the patient's shower, or tap if a shower was not present. Shower heads were not removed for water sampling. At

least 200 mL of water was collected into a vessel containing sodium thiosulfate as a neutraliser. In duplicate, 100 mL of water was filtered through a 0.45 µ filter and the filters placed onto CLED plates and cetrimide agar. Plates were incubated at 37°C for 48 h and the number of organisms per 100 mL quantified.

For storage and DNA extraction a single colony was purified from the primary culture plate. When different colony morphologies were observed, a single colony from each type was purified. Additionally, for a randomly selected water sample, 24 colonies were individually picked from one water-filter primary microbiological plate for sequencing. Isolates were stored on Biobank beads at −20°C prior to DNA extraction. Organisms were resuscitated on CLED agar plates and genome DNA either extracted directly using the MOBIO UltraClean Microbial DNA Kit, or from overnight LB broth culture using a Qiagen Genomic-Tip 100G.

DNA extraction and sequencing

Genomic DNA was prepared from single colony picks using the MOBIO Ultraclean microbial kit (MOBIO, Carlsbad, USA). 1 ng input DNA, as quantified by Qubit (Life Technologies, Carlsbad, USA) was used to prepare genomic libraries for sequencing using the Illumina Nextera XT DNA sample kit as per manufacturer's protocol (Illumina, San Diego, USA). Libraries were sequenced on the Illumina MiSeq using a paired-end protocol resulting in read lengths between 150 and 300 bases. A single additional sample, isolate 910, was chosen as a representative member of Clade 5 for long-read sequencing. DNA from this sample was fragmented using a Hydroshear (Digilab, Marlborough, Massachusetts, USA) using the recommended protocol for 10 kb fragments and further size-selected on a BluePippin instrument (Sage Science, Massachusetts, USA) with a 7 kb minimum size cut-off. The library was sequenced on two SMRT Cells using the Pacific Biosciences RS II instrument at the Norwegian Sequencing Centre, Oslo. C4-P2 chemistry was chosen because it favours long, more accurate reads for *de novo* assembly.

Stool PCR

For simple presence/absence detection of *P. aeruginosa* in stool samples using PCR, a stool sample was collected into a stool collection tube containing stool DNA stabiliser. Total DNA was extracted using the PSP Spin Stool DNA Plus kit (Stratec Molecular). PCR amplification of species specific regions of the 16S rDNA gene was carried out using primers PA-SS-F: GGGGGATCTTCG GACCTCA and PA-SS-R: TCCTTAGAGTGCCCACCCG [12] in the following conditions: 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's using BIOTAQ DNA Polymerase and buffer set. After initial denaturation at 96°C for 2 min, 30 cycles of 96°C for 30 s, 62°C for 30 s and 72°C for 30 s were completed with a final extension of 72°C for 5 min. Products were visualised for size on an 1.5% agarose gel.

Bioinformatics methods

Illumina MiSeq reads from each isolate were adapter and quality trimmed before use with Trimmomatic.[27] Phylogenetic reconstruction of isolates sequenced in this study were combined with data from a global collection of 55 *P. aeruginosa* strains collected world-wide which have been previously analysed by Stewart *et al*.[28] For each of the published strains, 600 000 paired-end reads of length 250 bases were simulated using wgsim (https://github.com/lh3/wgsim) from the complete or draft genome assembly deposited in Genbank. Read sets were mapped against the *P. aeruginosa* PAO1 reference genome using BWA-MEM 0.7.5a-r405 using default settings.[29] Single nucleotide polymorphisms were called using VarScan 2.3.6 and filtered for regions with an excessive number of variants. These may represent regions of recombination, misalignments or strong Darwinian selection.[30] FastTree (V2.1.7) was used for phylogenetic reconstruction. This software estimates an approximate maximum-likelihood tree under the Jukes-Cantor model of nucleotide evolution with a single rate for each site (CAT).[31] Trees were drawn in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

For *in silico* MLST prediction, trimmed reads were assembled de novo using Velvet [32] with a k-mer size of 81 and searched using nucleotide BLAST against the multilocus sequence database downloaded from the pubMLST website on 5 August 2013 (http://pubmlst.org/paeruginosa/).[33] For Clade E isolates, in order to exhaustively search for discriminatory mutations, a nearly complete reference genome was generated by de novo assembly using Pacific Biosciences sequencing data. Reads were assembled using the 'RS_HGAP_Assembly.3' pipeline within SMRT Portal V2.2.0. Illumina reads from the same sample were mapped to this draft genome assembly in order to correct remaining indel errors in the assembly using Pilon (http://www.broadinstitute.org/software/pilon/). Isolates belonging to each clade were mapped individually against either the PacBio reference (Clade E) or *P. aeruginosa* PAO1 (NC_002516; Clades C, D and G).

Variants (single nucleotide polymorphisms and short insertion-deletions) were called using SAMtools mpileup and VarScan with an allele frequency threshold of 80%.[30] Non-informative positions and regions of putative recombination were removed, the later with a variant density filter of more than 3 SNPs every 1000 nucleotides. Analysing samples in each clade individually maximised the number of variants detected by reducing the likelihood of the position being uncovered by a subset of samples. From these variants fine-grained phylogenetic trees were reconstructed for each clade using FastTree. The scripts used to perform this analysis are available at http://www.github.com/joshquick/snp_calling_scripts. Approximate-maximum-likelihood phylogenetic trees were generated using FastTree and visualised in FigTree. For whole-genome shotgun metagenomics analysis, reads were analysed using the Kraken taxonomic classifier

software with the supplied *minikraken* database.[34] Reads from the metagenomics data set were aligned to *P. aeruginosa* Clade E as in the previous section and phylogenetic placement was carried out using pplacer in conjunction with FastTree.[35] Sequence data is available from the European Nucleotide Archive for the Illumina data (ERP006056) and the corrected Pacific Biosciences assembly (ERP006058).

RESULTS

Study results

Recruitment lasted a period of 300 days, ending according to protocol after the enrolment of 30 screening patients. In total, we detected *P. aeruginosa* in five patients. Of these patients, three had *P. aeruginosa* detected only in burns wound swabs, one had *P. aeruginosa* detected in their burns wound and in their urine, and one had *P. aeruginosa* in their sputum. One additional eligible patient did not consent to enter the study and was excluded. The average age in the study group was 41 years. Males predominated with a male-to-female ration of 2.3:1. Flame burns were the most common mechanism of injury, followed by scalds and mixed flame/flash injuries. The average burn size of the study group was 12.5% of the TBSA and 27% of patients sustained an inhalation injury. Eight patients required admission to intensive trauma unit (ITU) and the majority required surgical treatment of their burns with excision and skin grafting (80%). A large majority of the study group (83%) received shower cart hydrotherapy as a routine part of their wound management to encourage healing through wound debridement and decontamination. The average length of hospital stay (LOS) was 17 days and taking into account burn size, the average was 1.4 days per % TBSA.

The water and environment in burns and critical care units are frequently colonised by *P. aeruginosa*

A total of 282 water and environmental samples were screened for *P. aeruginosa* of which 39/78 (50%) were positive in water samples, 25/96 (26%) were positive from the wet environment and 7/108 (6%) were positive from the dry environment. A total of 86 genome sequences were generated from the 71 positives, as in some cases multiple colony picks were sequenced. Seventy-eight patient samples were screened for *P. aeruginosa* of which 39 (50%) were positive. A total of 55 genome sequences were generated, as in some cases multiple colony picks were sequenced. In total, 141 genomes were sequenced; water and environmental (n=86) and patient (n=55). Genomes were sequenced to a mean coverage of 24.4×, with the minimum coverage of a sample being 14× and highest 64.7×.

When placed in the context of a global collection of *P. aeruginosa* strains, phylogenetic reconstruction demonstrated isolates in our study fell into eight clades (figure 1A). As has been reported previously, there was no strong association between ecological context and position in the phylogenetic tree.[28] Isolates in this study are most closely related to strains from a variety of settings. The majority of isolates (52%) belong to Clade E (figure 1B), whose nearest sequenced relative is the Liverpool Epidemic Strain, a clone often isolated from patients in the UK and Canada with cystic fibrosis. [36 37] Isolates from Clade E were found in the burns unit's water and the ward environment, as well as from two patient's wounds. However it was never detected in the critical care unit. Clade E was detected throughout the study in a total of 10 different rooms (figure 2).

Inferring potential transmission events by WGS

Microevolutionary changes occurring over rapid timescales (ie, days to months) have been used to detect potential chains of transmission in hospital and community outbreaks.[19–21 38 39] The number of distinct mutations between given isolates has been used to infer whether transmission events are likely to have occurred. Such inferences are aided by prior knowledge of mutation rates in similar populations. Two patients (1 and 4) in our study both had *P. aeruginosa* from Clade E isolated from their wounds. These isolates had an indistinguishable genotype from those present in water and the environment of the room they were nursed within (figures 1C and 3). This genotype was detected in the patient's shower water after initial patient screening, during screening of the second patient admission, twice during the second patient's stay and then 127 days later (days 27, 65, 89 and 216, respectively). When water isolates were positive, the genotype was also detected in wet environment sites (shower drain, shower rosette and patient's trolley) on the same days.

Patient 5 was nursed on the critical care unit due to concomitant medical problems. *P. aeruginosa* belonging to Clade G was isolated from sputum during this time. Identical genotypes were detected contemporaneously in the water from the associated sink and sink tap handle (see online supplementary appendix 4).

Two further patients (patients 2 and 3) were positive for *P. aeruginosa*. Isolates from these patients belonged to Clade C and D, respectively. Neither clade was ever isolated from hospital water. In both cases, identical genotypes were detectable in the environment associated with the patient but these were not detected before or after the patients' stay, indicating that the environment was not persistently contaminated. During the course of patient 3's stay, the dry environment such as the bedside table was contaminated, as was the patient's door handle and shower chair. However, after patient discharge, the strain associated with this patient was never seen again during the course of the study in any location.

WGS permits source tracking of *P. aeruginosa* to individual water outlets

WGS has been reported previously for source tracking, but never for the detection of transmission events from

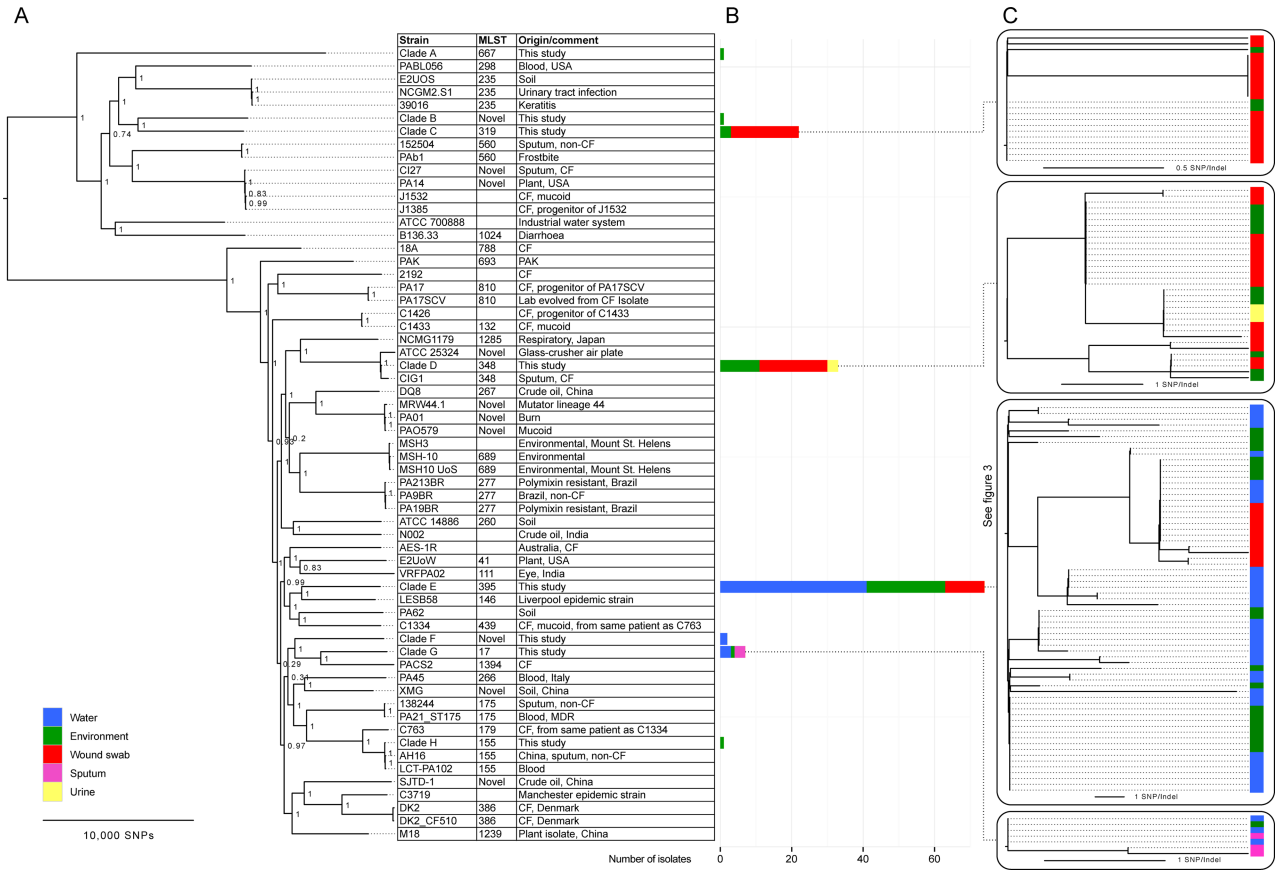

Figure 1 An overview of all samples collected during the study in global phylogenetic context with other sequenced strains of *Pseudomonas aeruginosa* from the set of Stewart *et al*.[28] Samples collected in this study are widely dispersed in the tree, which contains isolates from different environments (A). Bar plots indicate the numbers of each type of sample collected (B). Microdiversity within each clade is shown, with the colour bar indicating the source of each sample (C).

hospital water.[40] Phylogenetic reconstruction within Clade E, the most commonly detected water clone demonstrated additional diversity within this clone, with a total of 46 mutations detected an average genetic distance between isolates of 4.1 mutations (figure 3). The reconstruction demonstrated clear evidence of clustering of genotypes both by room and outlet (figure 3). When *P. aeruginosa* was detected in the wet environment (eg, shower rosettes and drains) these genotypes were most often identical to those found in water, indicating that the water was likely the ultimate source of that clone. Genotypic variation was seen between outlets within the same room. For example, tap water sampled from room 11 had a distinct genotype from that sampled from shower water in the same room and this was consistently found over multiple samplings. Notably, isolates from two patients fell within the cluster originating from shower water, indicating that shower hydrotherapy was the most likely source of infection. Two plasmids (designated pBURNS1 and pBURNS2) were detected in this study set, which both demonstrated geographical clustering, with pBURNS1 only being detectable in isolates from room 8 and pBURNS2 only being detectable in isolates from the shower water in room 9.

Rapid evolution of antibiotic resistance associated with treatment

P. aeruginosa is commonly associated with antibiotic resistance due to a number of predisposing features including intrinsic resistance, a repertoire of efflux pumps and antibiotic-inactivating enzymes including β-lactamases.[41] Three infected patients (2, 3 and 5) received antibiotic therapy, and in each case this was associated with the development of resistance to at least one therapeutic agent. Associated mutations were detected that were either partially or fully explanatory of the phenotype (online supplementary appendix 12).

Patient 2 was treated with ciprofloxacin, nitrofurantoin and vancomycin (see online supplementary appendix 11 for full details). Eight of 21 (38%) tested isolates from this patient were ciprofloxacin resistant. Seven of eight isolates (88%) of the ciprofloxacin-resistant strains were distinguishable from the other isolates by a single SNP in *mexS* (annotated as PA2491 in *P. aeruginosa* PAO1; see online supplementary appendix 1 and 7). This SNP was predicted to result in a non-synonymous amino acid substitution. Disruption of this gene has been shown to cause increased expression of the mexEF-oprN multidrug efflux pump, associated with resistance to quinolones.[42]

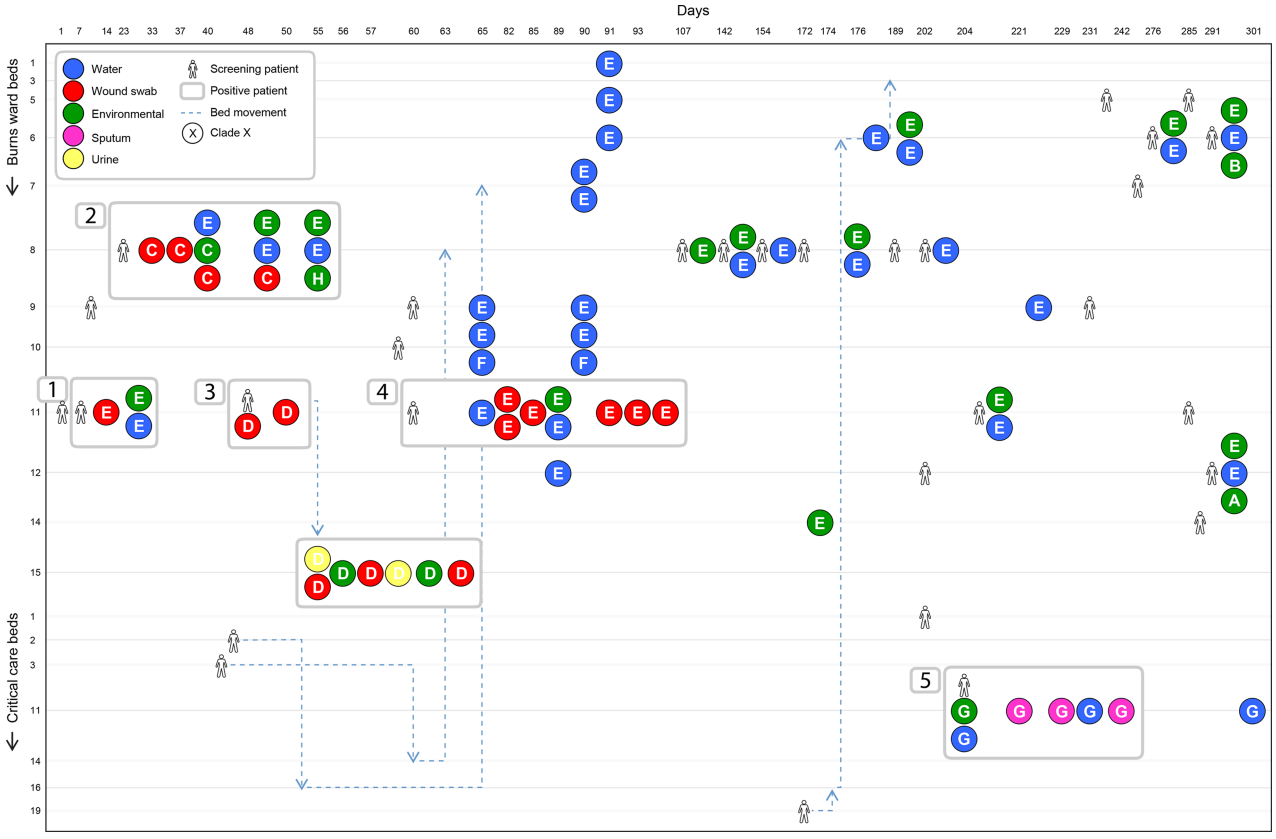

Figure 2 A schematic view of the 300-day study of *Pseudomonas aeruginosa* in a burns centre and critical care unit. Time in days is shown along the x axis with bed numbers in the critical care unit and burns unit along the y axis. Each circular icon indicates a positive isolate of *P. aeruginosa*. The icon's logotype indicates which environment it originated from (wound, urine/sputum, environment or water). The filled colour of the icon indicates the clade it belongs to. Patient icons represent the enrolment of a screening patient into the study and their location. Patient movements around the hospital are noted by dotted lines. The five patients infected with *P. aeruginosa* are denoted by rounded boxes. Boxes are coloured according to the patient number. In the event two or more isolates of the same source and clade were collected on the same day, these have been collapsed into a single circular icon.

Patient 3 was not treated with antibiotics, but isolates associated with this patient demonstrated differences in resistance to timentin and piperacillin-tazobactam. These changes were associated with non-synonymous mutations in *gacA*, the response regulator of the GacA/GacS two-component system and in *lasR*, a transcriptional activator required for transcription of elastase and LasA protease (online supplementary appendices 2 and 8).

Patient 4 was treated with meropenem, piperacillin/tazobactam, flucloxacillin and colistin. Five isolates collected 10–18 days after initiation of meropenem showed resistance to imipenem and intermediate resistance to meropenem (see online supplementary appendix 3 and 9). The most likely mutation responsible for this phenotype was detectable in two isolates, both of which had a frame-shift mutation in the gene coding for the membrane porin OprD.[43]

Patient 5 had a prolonged stay in ITU and had multiple medical problems including *A. baumannii* infection and was treated with nine antibiotic agents including ciprofloxacin, meropenem and piperacillin-tazobactam. Serial isolates from this patient demonstrated the stepwise acquisition of two mutations (online supplementary appendix 4). The first was in *nalC*, a probable repressor of the TetR/AcrR family (online supplementary appendix 10).[44] On inspection of the sequence alignment in this region, a large deletion of 196 nucleotide bases was seen compared to the reference PAO1 strain. This mutation was seen in association with full resistance to piperacillin-tazobactam, ceftazidine, aztreonam, meropenem and intermediate resistance to ciprofloxacin. This deletion is likely to result in over-expression of efflux pumps involving the *mexAB-oprM* operon.[44 45] Ciprofloxacin resistance in a later isolate corresponded to the stepwise acquisition of a second mutation. This mutation is predicted to affect the well-studied DNA gyrase subunit A gene (*gyrA*) which is strongly associated with ciprofloxacin resistance.[46]

Confirmation of *P. aeruginosa* genotypes in biofilms by whole-genome metagenomic shotgun sequencing

P. aeruginosa is able to produce and survive in biofilms. Plumbing parts such as flow straighteners, shower rosettes,

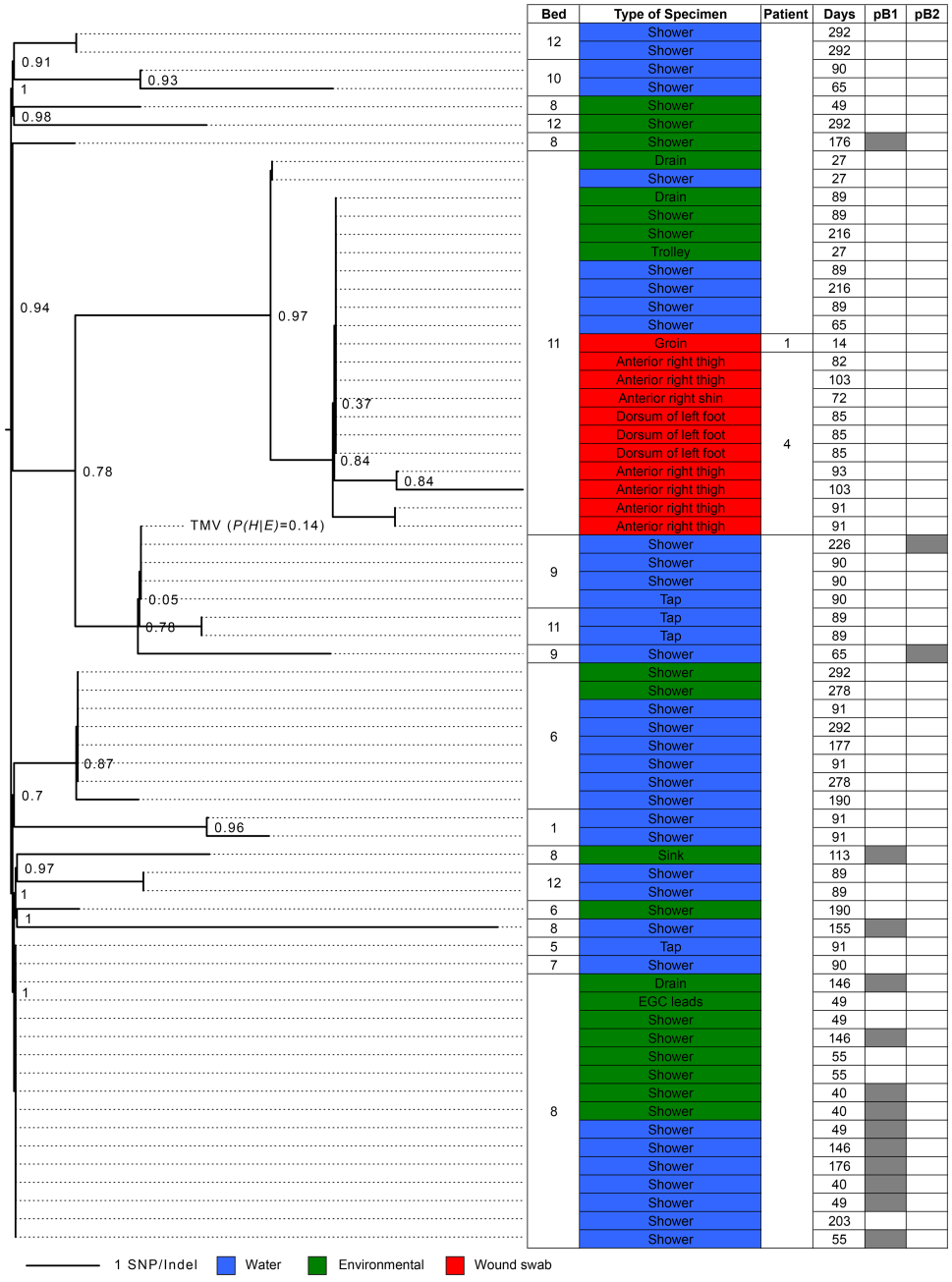

Figure 3 The high-resolution phylogenetic reconstruction of Clade E isolates. This demonstrates the clustering of genotypes by bed space. Patient associated samples are contained within a room 11 clade. This clade contains water samples from the shower and environmental samples from the shower, drain and trolley. The water samples from the room 11 tap are in a distinct clade, indicating the biofilm within the tap has a distinct genotype to the shower. This suggests environmental contamination was more likely to arise from contaminated shower water than tap water. Details of sampling site, days since start of study and presence of pBURNS plasmids are also shown. The likely phylogenetic position of *Pseudomonas aeruginosa* detected in a biofilm from a thermostatic mixer valve is shown in the clade associated with room 9 and indicated 'TMV'.

flexible hoses, solenoid valves and thermostatic mixer valves (TMV) are particularly at risk of biofilm formation due to factors including surface areas, convoluted designs and inadequate pasteurisation.[47] To confirm the presence of *P. aeruginosa* in water fittings associated with rooms on the burns unit, we obtained a TMV removed by the hospital estates team from the shower in room nine as part of compliance with UK guidelines for managing *P. aeruginosa* in hospitals. On visual inspection, a biofilm was present which was scraped from the surface with a sterile scalpel. DNA from this biofilm was extracted for whole-genome shotgun sequencing. The majority of reads did not map to any known bacterial taxa. The most abundant taxon identified was *P. aeruginosa* (3%). Subsequent alignment to the *P. aeruginosa* Clade E reference covered 94% of the 6.3 million base reference genome at a median coverage of

5×, confirming that reads were correctly classified to this species and not other environmental *Pseudomonas* species. Alignment to the *P. aeruginosa* Clade E reference genome followed by phylogenetic placement of reads demonstrated that it fell into the same clade as previously recovered isolates from the shower or tap in room 9 (indicated on figure 3, and in online supplementary appendix 6).

DISCUSSION

The hospital environment has been intimately linked with *P. aeruginosa* infection for over 50 years yet hospital acquisitions, clusters and outbreaks remain a common occurrence and understanding precise routes of transmission can be difficult.[47 48] Our results demonstrate that, even in a new hospital, *P. aeruginosa* can become rapidly endemic in hospital plumbing. Furthermore, by linking *P. aeruginosa* genotypes recovered from patients to specific individual water outlets, we offer compelling evidence of unidirectional transmission from water to patients. Further, by sequencing of a biofilm identified in a TMV from a hospital water system, we can identify the likely common source of genotypes found in water and in the hospital environment.

Our results suggest that use of WGS can reduce ambiguity about potential transmission events in hospitals and consequently inform infection prevention efforts about the direction and sequence of transmission. Typing schemes such as MLST and PFGE are much lower resolution methods and would not be able to provide sufficient information to permit such inferences to be made. It is notable that the burns unit was colonised by a single clone, meaning that it was very unlikely that water outlets at each bed space were colonised as a result of transmissions from the patient or environment. For this to happen would require multiple transmission events from separate patients with the same clone, for which there is no evidence. Instead we speculate that this clone was introduced to the hospital associated with its commissioning. One hypothesis is that particular plumbing fittings, that is, the TMV may have been colonised simultaneously by a clone circulating in water. Clade E (ST395) has been frequently reported associated with water, so this remains a possibility. [49 50] However, it is possible that plumbing fittings are installed 'pre-seeded' with *P. aeruginosa* as has already been proposed by Kelsey.[3 5 47] Investigation of an outbreak in Wales implicated new plumbing parts as a potential source of *P. aeruginosa*. New plumbing components are often tested by companies prior to their supply and it is possible they were contaminated prior to distribution. The limited amount of diversity (average 4 SNPs) seen within this clade is consistent with a single founding genotype coinciding with the opening of the burns unit, based on estimates from a previous study using WGS which reported that mutations accumulate at a rate of approximately one every 3–4 months in a hospital-associated clone.[51] However, our results suggest that our isolates accumulate mutations even more slowly. This may be due to reduced growth rates in nutritionally-poor biofilms.[52]

It is notable that antibiotic resistance to multiple first-line agents developed rapidly in response to therapy. These results underline the importance of selecting appropriate antibiotic therapy in *P. aeruginosa* infections. It is reassuring however that antibiotic resistance genotypes selected *in vivo* did not show evidence of persistence in the ward environment or transmission to other patients.

Our study has certain limitations. Based on a previous audit, we expected around one-third of patients screened for *P. aeruginosa* would develop colonisation or clinical infection. In fact, only 5 out of 30 of patients were colonised. This may have been related to guidance and engineering interventions being put in place during the study as detailed in national guidance issued while this study was on-going. In addition, infection control policies were revised to address control of an outbreak of a multidrug resistant *A. baumannii* in this same burns unit. Following these interventions, only 1 of the last 20 patients recruited was infected with *P. aeruginosa* which may demonstrate the importance of national guidance in reducing transmissions.

By focusing on burns patients who receive hydrotherapy, our study population were at extremely high risk of waterborne infection. In other patient groups it may be that alternative routes of transmission including cross-infection or endogenous carriage play a more important role. Our results suggest that our burns unit is endemically colonised with a distinct clone of *P. aeruginosa* that may have been imported coinciding with the opening of the hospital. Other intensive care units, particularly those which have been open for longer may harbour a greater diversity of *P. aeruginosa* as a result of increased opportunities for clones to be imported.

One potential application for WGS in infection control would be to determine whether cases are as a result of water transmission, or represent sporadic clones originating from the wider environment. Despite improved guidance concerning improved engineering infection control practices and the introduction of the water safety group in the UK, it may not be realistic to eliminate *P. aeruginosa* from hospitals entirely. In augmented care units such as ITUs, burns units and neonatal wards where *P. aeruginosa* poses a significant risk to vulnerable patients, the increased resolution offered by WGS will justify its use, particularly as the costs continue to fall.

In conclusion, we have identified through WGS clear evidence for transmission of *P. aeruginosa* from specific water outlets to burns patients and offer a forensic-level framework for dealing with outbreaks linked to hospital water. We expect WGS will continue to make inroads into clinical microbiology and become a vital tool for tracking *P. aeruginosa* in the hospital environment, helping inform targeted control measures to help protect patients at risk of infection.

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Contributors MJP, NSM and BO conceived the study. CMW and AB enrolled patients into study and collected samples. NC collected environmental and water samples. NC, CC and MN processed samples and performed microbiology. NC, CC and JQ did sequencing. JQ, NC, CMT and NJL analysed the data. NJL, NC, JQ, MJP and BO wrote the paper. All authors commented on the manuscript draft.

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Competing interests None.

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3 Rapid draft sequencing and real-time nanopore sequencing in a hospital outbreak of Salmonella

3.1 Author contributions

SC and **JQ** performed sequencing. **JQ**, PA, TD, ER, PH and NJL analysed data. NJL, TD, PH and MW contributed reagents. NJL, PH and **JQ** wrote the manuscript. All authors read and approved the final manuscript.

3.2 Author contributions (additional detail)

JQ generated the sequencing libraries and wrote the MiSeq draft sequencing scripts. JQ performed the phylogenetic analysis, the quality comparison for draft sequencing and wrote the scripts to perform the streamed genotyping and the pplacer analysis. JQ drafted figures, tables and contributed to the writing of the manuscript.

3.3 Abstract

In this study we investigated the use of both the MiSeq and the MinION to provide clinically relevant information during an ongoing local hospital outbreak of *Salmonella enterica* Serovar Enteritidis. During the outbreak we sequenced 16 isolates using a 6 hour MiSeq 'draft sequencing' protocol we developed to quickly rule isolates in or out of the outbreak. Later we sequenced two isolates on the MinION, one known to be from

the outbreak and one not. In order to simulate real-time data, the reads were analysed as they would have become available in real time i.e. at ten minute intervals after the start of the run. We demonstrated two new analysis approaches; the first able to unambiguously identify the species as *Salmonella enterica* within 30 minutes and the second able to assign one sample to the main hospital cluster within 100 minutes and the other to a different cluster containing a mixture of phage types none of which were the same as the hospital cluster (14b) within 120 minutes. Surveillance sequencing of foodborne pathogens is an exciting development pioneered by the Food and Drug Administration (FDA)[68] and Public Health England (PHE). Integrating our data with these resources allowed us to place the outbreak in a national and international context.

3.4 Published manuscript

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Rapid draft sequencing and real-time nanopore sequencing in a hospital outbreak of *Salmonella*

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Abstract

Background: Foodborne outbreaks of *Salmonella* remain a pressing public health concern. We recently detected a large outbreak of *Salmonella enterica* serovar Enteritidis phage type 14b affecting more than 30 patients in our hospital. This outbreak was linked to community, national and European-wide cases. Hospital patients with *Salmonella* are at high risk, and require a rapid response. We initially investigated this outbreak by whole-genome sequencing using a novel rapid protocol on the Illumina MiSeq; we then integrated these data with whole-genome data from surveillance sequencing, thereby placing the outbreak in a national context. Additionally, we investigated the potential of a newly released sequencing technology, the MinION from Oxford Nanopore Technologies, in the management of a hospital outbreak of *Salmonella*.

Results: We demonstrate that rapid MiSeq sequencing can reduce the time to answer compared to the standard sequencing protocol with no impact on the results. We show, for the first time, that the MinION can acquire clinically relevant information in real time and within minutes of a DNA library being loaded. MinION sequencing permits confident assignment to species level within 20 min. Using a novel streaming phylogenetic placement method samples can be assigned to a serotype in 40 min and determined to be part of the outbreak in less than 2 h.

Conclusions: Both approaches yielded reliable and actionable clinical information on the *Salmonella* outbreak in less than half a day. The rapid availability of such information may facilitate more informed epidemiological investigations and influence infection control practices.

Background

Outbreaks of *Salmonella* from contaminated food are frequently reported in the community, with 1.2 million cases estimated to occur in the US each year [1]. In a population-based study in the UK in 2008–2009, there were >38,600 estimated cases of salmonellosis and 11,300 patients presenting to a primary care physician [2]. Hospital outbreaks of *Salmonella* may result from patient-to-patient spread and can be lethal in vulnerable patients [3–5]. An example is the hospital outbreak at Stanley Royd Hospital in the UK which led to the deaths of 19 patients and a public inquiry [2, 6]. We recently detected a cluster of more than 30 cases of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) over a 3-week period at one of three hospital sites in our hospital organisation and from the community. This was against a typical background incidence of five to eight cases per month of all *S. enterica* isolates in the area served by our hospital. Initially a small number of seemingly unrelated, presumed community-acquired cases were detected on different wards but subsequently a larger number of long-term inpatients on two adjoining wards were affected suggesting the possibility of spread within the hospital. Simultaneously, an increase in community isolates was also detected. At first, it was unclear whether hospital cases were reflecting multiple imports from a community outbreak or spread within the hospital or both. Due to the explosive nature of the outbreak, coupled with uncertainty about the source, a

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rapid response was required to ensure that infection control measures were appropriately targeted. Outbreak investigations are aided by rapid availability of whole-genome sequencing (WGS) data, as this provides the greatest level of discrimination between isolates when compared to traditional typing methods such as phage typing, multilocus variable number tandem repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE) [3–5, 7]. The Illumina MiSeq sequencing platform has emerged as the gold standard for WGS investigations of outbreaks, but results may not be available for as long as 3 working days, depending on the protocol used [8–10]. A number of studies have evaluated the utility of WGS for typing *S. enterica* isolates; however, to the authors' knowledge, this is the first use of prospective typing of this organism during an outbreak. Rapid availability of accurate typing results is critical to effective outbreak control. We therefore devised a novel rapid draft sequencing protocol on the MiSeq generating results in under 6 h following library preparation. At the time of the outbreak we were testing a portable, handheld, 'USB stick', whole-genome sequencer, the MinION (Oxford Nanopore Technologies, UK), as part of their early access programme. We wished to see what role this technology might play in the management of future outbreaks.

Our initial goals when performing sequencing prospectively were: (1) to determine if cases in the hospital were from the same strain as those circulating in the community, and to discriminate outbreak cases from normal background *S. enterica* strains; (2) to determine whether there was evidence of a super-shedder patient or specific breakdown in infection control practices; (3) to help link cases to a primary source (for example, person or food) and to compare to previous outbreak strains; and (4) to integrate these results with national surveillance data.

Results and discussion

Epidemiological investigation

In total, 43 isolates of *S.* Enteritidis were identified in the study period (1 to 24 June) from inpatients, community samples from general practitioners and from environmental isolates. Hospitalised cases were only identified at one hospital site in the group of three hospitals. The same hospital food is distributed to all three hospital sites from a single, central kitchen processing unit where hot food is twice-cooked to standards that would kill salmonellae. All microbiological testing of hospital food was negative for *Salmonella*. The environmental swabs from affected wards were all negative apart from one isolate of *S.* Enteritidis recovered from the outside door seal of a food regeneration trolley. This proved to be of the outbreak type. Four separate colony picks were sequenced from this culture. Isolates from staff were sent to the reference laboratory by another laboratory in a different city 14 miles away. These were detected in faecal samples submitted by general practitioners and were found to belong to staff at our hospital working on the affected wards. The first 16 samples, of which six cases had onset dates compatible with community acquisition, were available for sequencing on 10 June and 13 samples sequenced successfully. These were subsequently shown to be distinct from other isolates recently sequenced by national surveillance and were identical to each other, apart from three cases that each had one SNP difference (Fig. 1, Panel a).

Sequencing of isolates from two early patient cases on 3 and 4 June showed them to be identical. As both patients had been hospitalised for longer than the *Salmonella* incubation period this was strongly suggestive of hospital acquisition. There were nine other cases on or prior to 4 June, which together with the typing data helped to inform further infection control actions. All symptomatic patients were isolated and the two wards were closed. Deep cleaning was undertaken with vaporised hydrogen peroxide sterilisation. Four isolates from the later part of the outbreak were identified by SNP typing to be unrelated to the outbreak type. Two of the four isolates were from young children who had recently returned from separate holidays in Egypt. These isolates were different to each other but one was identical to another isolate from a child of similar age who had not travelled abroad, which prompted further epidemiological investigation. It emerged that the two children attended the same nursery in a town just outside the city in which the hospital outbreak had been detected, strongly suggesting transmission had occurred within the nursery (Fig. 1).

The earliest date of onset was 25 May and the last 8 July. A total of 37 cases with the outbreak strain were identified of which six had no connection with the hospital, eight were staff members and three were asymptomatic carriers identified by screening patients on outbreak wards. Comparison of the outbreak genome sequence with the Public Health England database of strains from the whole of England and Wales suggested a very close relationship to six isolates from London, Bedford and Northampton. Further epidemiological investigation of these cases found no link to Birmingham or a foodstuff. The outbreak strain was PT 14b and multi-locus variable number tandem repeat (MLVA) type 2-11-9-7-4-3-2-8-9, an uncommon type for PT 14b strains.

Rapid draft sequencing on the Illumina MiSeq

As an initial response to the outbreak, isolates from 16 patients were sequenced overnight on the Illumina MiSeq on 12 June 2014 in order to generate results for

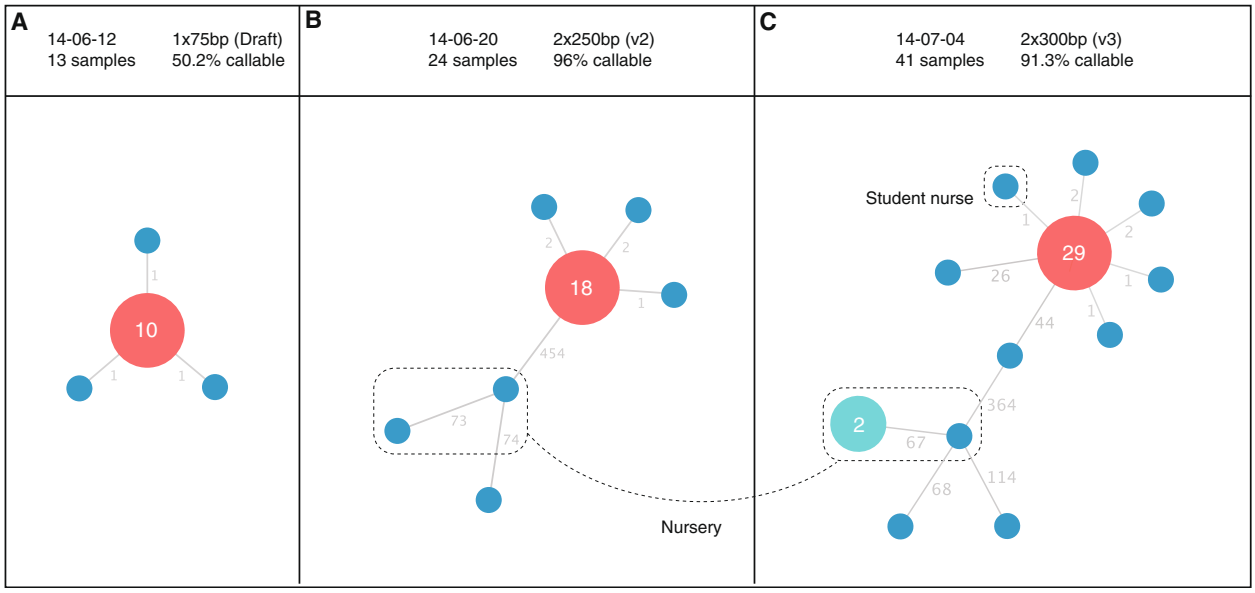


Fig. 1 Phylogenetic reconstruction of the outbreak, as sequencing data were generated at three time points during the outbreak (corresponding to Panels **a, b** and **c**). The red node demonstrates the most frequently occurring SNP type. Node labels show the number of isolates of this type. Blue nodes are singletons. Edge labels show the number of SNPs between isolates. The turquoise node shows a cluster of two identical isolates from cases associated with a nursery. The header bar shows the date of the sequencing, the Illumina sequencing protocol used, the number of samples in the dataset and the total percentage of the *S.* Enteritidis reference genome which could be used for SNP calling (core genome). Subtle differences in the number of SNPs between nodes on the tree may be seen, for example in the unrelated nursery isolates (6 SNPs different between Panel **a** and Panel **b**). These differences are attributed to differences in core genome size, that is, the number of positions in the reference genome used to generate the results. The precise core genome size figures are shown as a percentage in the panel headings

an infection control meeting the following day (results shown in Fig. 1 Panel a). To enable this, we devised a new draft sequencing protocol that reduced the run time of the MiSeq instrument to 6 h (contrasted with standard protocols which can take up to 55 h to complete). This was achieved by reducing the read length, cycle time and number of tiles imaged. Of the 16 isolates, 13 had a mean coverage depth of greater than 4× (mean 8×) and could be used for further analysis. Due to the lower coverage of strains, 50.2 % of the core genome was used to generate these results. Despite this, the results generated within 6 h were sufficient to conclude that the initial set of isolates were all part of the same outbreak (10/13 isolates were identical when analysing the core genome of *S.* Enteritidis, three other isolates each differed by 1 SNP). Later on, when standard protocol MiSeq (paired 250 or 300 bp) data were available as well as HiSeq data from PHE surveillance, we were able to compare these results to that of draft sequencing. We could then conclude that although genome coverage was lower, the rapid draft sequencing method was concordant with both slower methods (Fig. 1). The sequencing quality using the draft protocol was lower (median Q score 36 compared with 38 using the V2 and V3 protocols at cycle 75) (Fig. 2).

Retrospective evaluation of real-time nanopore sequencing

Two samples, one belonging to the outbreak and one unrelated were sequenced on the newly-available MinION from Oxford Nanopore Technologies. During the outbreak, we used an early version of the chemistry termed R6. However, results from this sequencing did not produce sufficient numbers of high-quality two-direction (2D) reads to be of use. In July 2014 R6 chemistry was replaced by R7, which we were able to evaluate retrospectively. The MinION is characterised by very long reads, which have a high error rate compared to the Illumina platform.

Nanopore sequencing results

In order to evaluate the potential benefits of real-time sequencing to enhance infection control procedures we analysed read sets as they would have become available in real time, that is, at 10 min intervals after the run had been initiated. The two samples were run on separate flow cells. The number of reads generated in the first 170 min were 2,865 (first flowcell) and 3,447 (second flowcell) with mean read lengths of 6,340 and 4,664 bp, respectively for each sequence library. The mean read accuracy, determined by counting all differences from

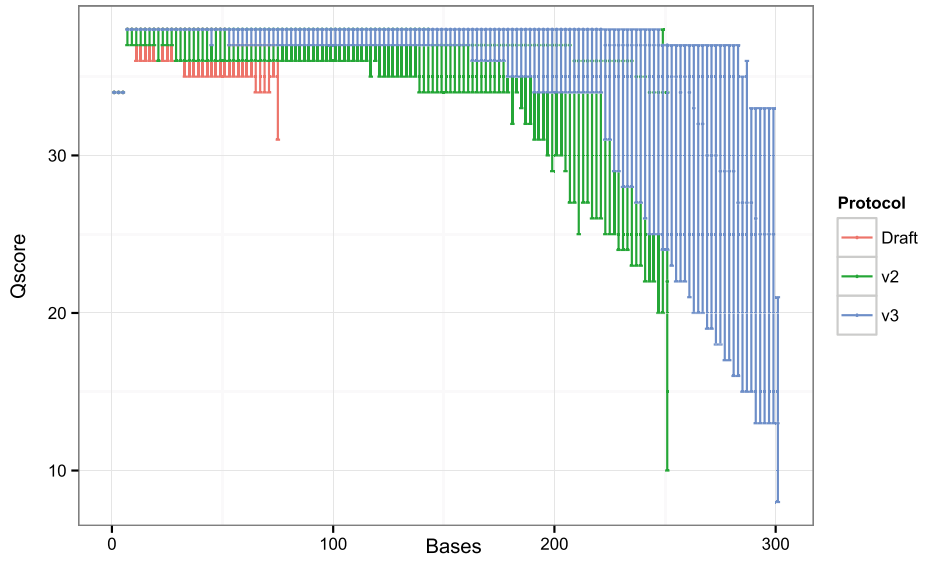


Fig. 2 Phred-scaled quality scores ($-10 \log_{10} P$) for Illumina sequencing demonstrating the impact of read length on read quality scores with the three Illumina MiSeq sequencing modalities used in this study. Red scores indicate results from the draft 1 × 75 base sequencing protocol, which shows minimally worse quality drop-off than running V2 (green, 2 × 250 base) or V3 chemistry (blue, 2 × 300) under standard conditions

the reference genome, was 72 % (first flowcell) and 73 % (second flowcell).

Real-time strain identification from nanopore reads

We found that in the two samples tested we could unambiguously identify the bacterial species *S. enterica* in less than 30 min (Fig. 3). Additionally, chromosomally encoded phage sequences were detectable and distinguishable between outbreak and non-outbreak strains within 50 min.

Genotyping from low coverage, error-prone data using phylogenetic placement

Genotyping accuracy improves as more sequencing data are available and a consensus sequence is formed (Table 1). Our genotyping protocol gets increasingly more precise as more reads are added, however recall stays relatively constant. Despite this, a phylogenetic placement method confidently assigned both the outbreak and non-outbreak strains to a clade of *S. enterica* containing the Gallinarum, Pullorum and Enteritidiis serovars very early on in the sequencing process. By 40 min it was possible to determine that the likely serovar was Enteritidis (Fig. 4). Once assigned to a serovar, further analysis could be restricted to a reference tree of *S.* Enteritidis strains. It was possible then to show that the outbreak strain unambiguously belonged to the main hospital outbreak cluster within 100 min of starting sequencing (Fig. 5). The non-outbreak strain was assignable to a clade containing several closely related strains (with a mixture of phage types, none of them PT 14b) within 120 min.

The availability of definitive typing data so early on in this outbreak enabled us to identify transmission between hospital wards and take rapid action to control spread. The appearance of cases in unrelated wards was puzzling initially, but WGS confirmed that the hospital SNP type was the same as that circulating in the community. This reassured the infection control team that there was not hospital-wide spread via some unknown vector. Preliminary food sample testing results were only available one day later. The finding of the outbreak strain on the door seal of the food trolley with the subsequent confirmation of cases in staff members supported the hypothesis that some local spread had occurred via the environment. Person-to-person spread may also have occurred. Towards the end of the outbreak the ability to rapidly identify cases not involved prevented much wastage of effort and resources. Remarkably we identified transmission of another strain of *S.* Enteriditis probably acquired in Egypt in a childcare group at a distant site because of the resolution of the typing information directing epidemiological investigations. Recent outbreaks of PT 14b strains in the UK have previously been associated with Spanish eggs, although the antibiotic resistance profile of the outbreak described here is different [11, 12]. Contemporaneously, outbreaks of *S.* Enteritidis PT 14b associated with consumption of eggs were reported in France, Austria and Germany, triggering an urgent outbreak investigation by the ECDC and EFSA [12]. Strains associated with this outbreak were of

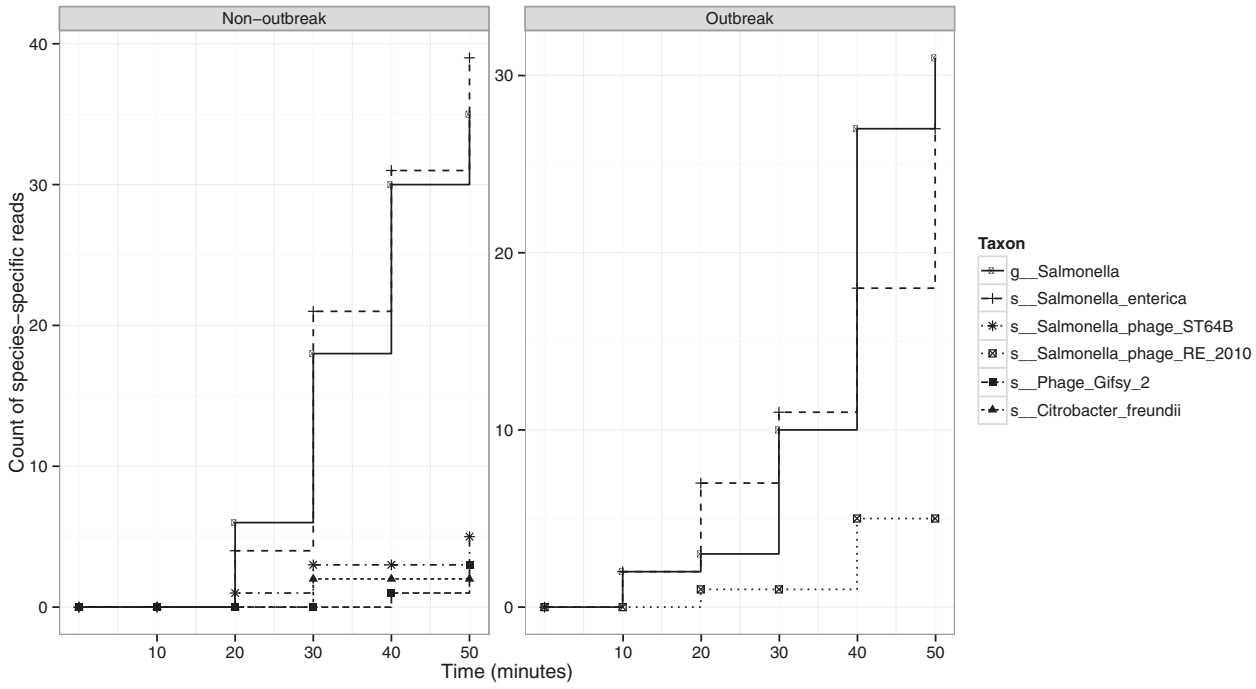


Fig. 3 Streaming taxonomic assignments from the Oxford Nanopore MinION are shown for an isolate belonging to the outbreak and an isolate not belonging to the outbreak. Assignments to *Salmonella* and *Salmonella enterica* are found within 10 min of starting sequencing for the outbreak strain and within 20 min for the non-outbreak strain. Phage-specific sequences are detected and are distinct between the non-outbreak and outbreak strain. The non-outbreak strain harbours *Salmonella* phage ST64B and Gifsy-2, whereas the outbreak strain harbours *Salmonella* phage RE2010

MLVA type 2-12-7-3-2 (using the 5-locus scheme), varying by a single locus from the isolates identified in this study. In these cases *S.* Enteritidis was isolated from eggs originating from a producer in Germany [12]. There is no definitive link between the outbreak reported in this study and the consumption of German eggs. However, the MLVA type in the European outbreak was also detected in the UK and eggs from the German producer are distributed for sale in the UK. Further whole-genome sequencing of European isolates is now being undertaken and may help determine whether the two outbreaks are linked to a common source.

This study illustrates a substantial future benefit from extremely rapid definitive WGS typing. The epidemiology of non-typhoidal *Salmonella* has changed significantly in the UK over the last decade and to a lesser extent in the rest of Europe [2, 13]. While non-typhoidal *Salmonella* rates have fallen overall, particularly in the UK following chicken flock vaccination, the proportion of disease caused by *S.* Enteritidis associated with travel has risen greatly. The ability to both identify serovars via deduced multi-locus sequence typing (MLST) and specific strains within a day of bacterial colonies being available will enable outbreaks to be investigated at a stage where accurate travel/food histories and possible person-to-person transmission can be elucidated and control measures introduced. We show that our method of rapid draft sequencing on the MiSeq is able to generate reliable results, despite generating reduced genome coverage. We anticipate this method will be of value to research groups needing to generate results in the timescale of a single working day, a considerable reduction compared to the standard protocols on this instrument.

The availability of national and international databases of sequencing data of food-borne pathogens marks an exciting step forward for epidemiological investigations. Surveillance by WGS has been pioneered by the US Food and Drug Administration, with results published online on the National Center for Biotechnology Information's GenomeTrakr service, an advantage of the portable, digital nature of genome data [8, 14]. In the UK, since 1 April 2014, Public Health England has been routinely sequencing all *Salmonella enterica* strains reported by hospitals and general practitioners to the *Salmonella* Reference Service, Colindale. Through integration with this dataset, we determined that the outbreak strains formed a distinct cluster, although this cluster varied by only a single core SNP from cases observed elsewhere in the UK.

We evaluated two sequencing methodologies in this study, both capable of providing rapid whole-genome sequencing information. The MinION senses individual

Table 1 Streaming alignment statistics from nanopore data

Flowcell	Time (m)	Reads	Bases	Positions	Missing bases	Covered (%)	True positive	True negative	False positive	False negative	Recall	Precision	Accuracy
Outbreak	60	920	5635627	7091	6463	8.86	10	617	0	2	0.83	1.00	0.09
Outbreak	120	2037	12853716	7091	4815	32.10	26	2237	7	7	0.79	0.79	0.32
Outbreak	180	3040	19297035	7091	3580	49.51	48	3436	13	15	0.76	0.79	0.49
Outbreak	240	3933	24900526	7091	2703	61.88	62	4291	17	19	0.77	0.78	0.61
Outbreak	300	4525	28614437	7091	2236	68.47	70	4736	25	25	0.74	0.74	0.68
Outbreak	360	5654	35848389	7091	1499	78.86	82	5454	26	31	0.73	0.76	0.78
Outbreak	420	6680	42498530	7091	1029	85.49	87	5914	25	37	0.70	0.78	0.85
Outbreak	480	7516	47950926	7091	749	89.44	94	6185	30	34	0.73	0.76	0.89
Outbreak	540	7913	50372188	7091	630	91.12	96	6300	29	37	0.72	0.77	0.90
Outbreak	600	8807	56254898	7091	463	93.47	103	6470	20	36	0.74	0.84	0.93
Outbreak	660	9666	61989423	7091	337	95.25	107	6588	22	38	0.74	0.83	0.94
Outbreak	720	10472	67171497	7091	267	96.23	111	6659	16	39	0.74	0.87	0.95
Outbreak	780	10833	69363106	7091	243	96.57	112	6686	16	35	0.76	0.88	0.96
Outbreak	840	11708	74625788	7091	191	97.31	117	6737	13	34	0.77	0.90	0.97
Outbreak	900	12479	79551399	7091	141	98.01	121	6780	16	34	0.78	0.88	0.97
Outbreak	960	13198	84228957	7091	120	98.31	124	6797	16	35	0.78	0.89	0.98
Outbreak	1020	13579	86600020	7091	107	98.49	125	6808	16	36	0.78	0.89	0.98
Outbreak	1080	14359	91437571	7091	90	98.73	126	6823	17	36	0.78	0.88	0.98
Outbreak	1140	15168	96646434	7091	74	98.96	124	6842	15	37	0.77	0.89	0.98
Outbreak	1200	15835	100970757	7091	70	99.01	123	6851	12	36	0.77	0.91	0.98
Outbreak	1260	16205	103367082	7091	63	99.11	124	6857	11	37	0.77	0.92	0.98
Outbreak	1320	16632	106040214	7091	60	99.15	125	6859	12	36	0.78	0.91	0.98
Outbreak	1380	17184	109618605	7091	56	99.21	125	6863	11	37	0.77	0.92	0.99
Outbreak	1440	17332	110500445	7091	55	99.22	124	6865	11	37	0.77	0.92	0.99
Non-outbreak	60	1268	5382184	7091	6372	10.14	1	717	2	0	1.00	0.33	0.10
Non-outbreak	120	2554	11567191	7091	4791	32.44	2	2284	15	0	1.00	0.12	0.32
Non-outbreak	180	3626	17058822	7091	3451	51.33	4	3607	29	1	0.80	0.12	0.51
Non-outbreak	240	4612	22004574	7091	2500	64.74	11	4545	32	4	0.73	0.26	0.64
Non-outbreak	300	5483	26582592	7091	1760	75.18	13	5281	35	3	0.81	0.27	0.75
Non-outbreak	360	6198	30340527	7091	1330	81.24	15	5705	40	2	0.88	0.27	0.81
Non-outbreak	420	6877	34040490	7091	985	86.11	16	6054	35	2	0.89	0.31	0.86
Non-outbreak	480	7522	37471113	7091	727	89.75	18	6306	37	4	0.82	0.33	0.89
Non-outbreak	540	8306	41387560	7091	552	92.22	18	6483	34	5	0.78	0.35	0.92
Non-outbreak	600	9032	45052523	7091	395	94.43	20	6643	28	6	0.77	0.42	0.94
Non-outbreak	660	9682	48325820	7091	304	95.71	20	6735	27	6	0.77	0.43	0.95
Non-outbreak	720	10262	51312827	7091	262	96.31	20	6783	21	6	0.77	0.49	0.96

Table 1 Streaming alignment statistics from nanopore data *(Continued)*

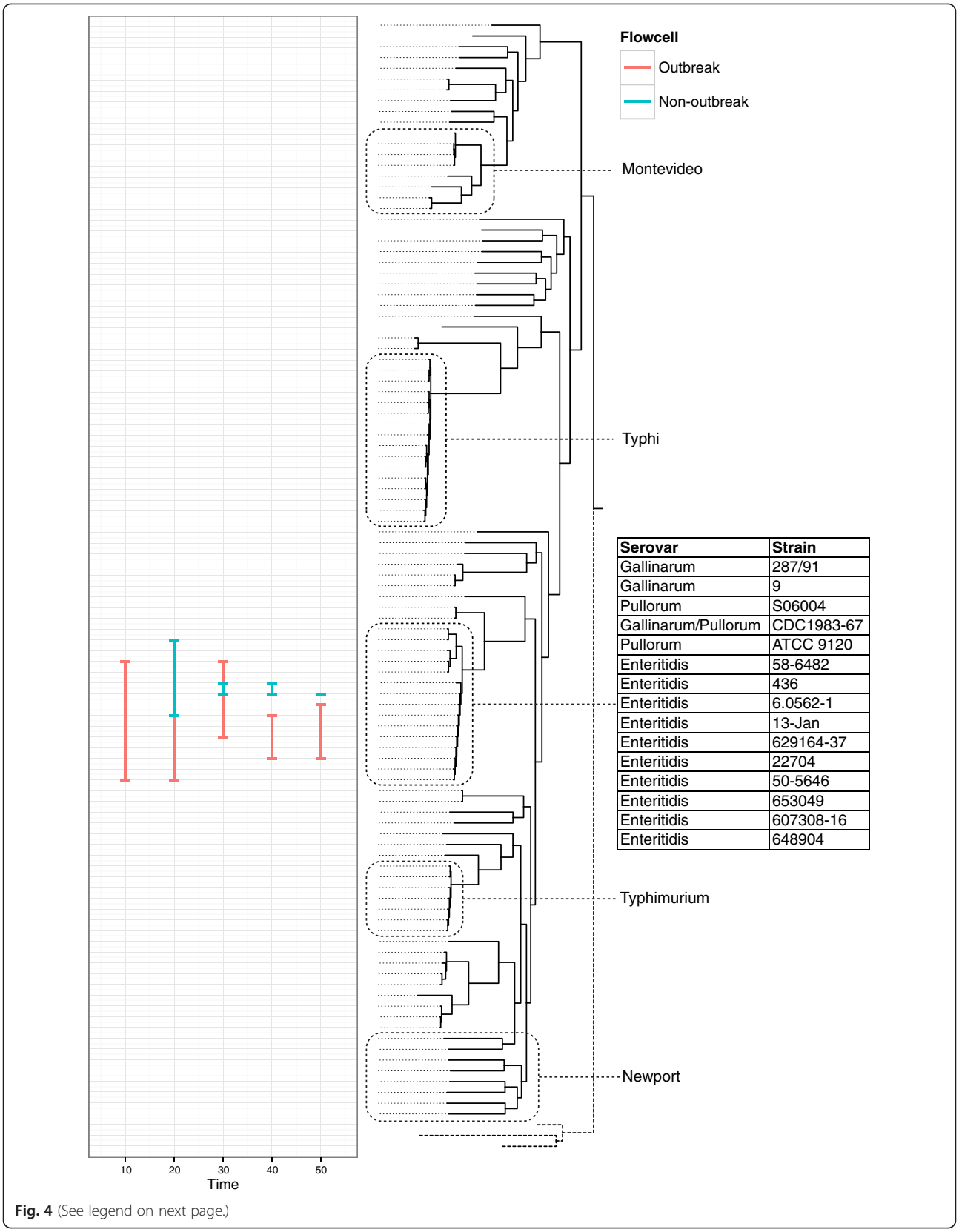
Non-outbreak	780	10845	54417219	7091	202	97.15	21	6845	18	6	0.78	0.54	0.97
Non-outbreak	840	11346	57135819	7091	178	97.49	22	6870	16	6	0.79	0.58	0.97
Non-outbreak	900	11793	59514439	7091	145	97.96	23	6898	18	8	0.74	0.56	0.98
Non-outbreak	960	12192	61590631	7091	111	98.43	22	6932	19	8	0.73	0.54	0.98
Non-outbreak	1020	12571	63597395	7091	99	98.60	22	6944	19	8	0.73	0.54	0.98
Non-outbreak	1080	12926	65415215	7091	87	98.77	21	6959	18	7	0.75	0.54	0.98
Non-outbreak	1140	13263	67138579	7091	71	99.00	22	6976	15	8	0.73	0.59	0.99
Non-outbreak	1200	13594	68911549	7091	62	99.13	22	6985	15	8	0.73	0.59	0.99
Non-outbreak	1260	13881	70408443	7091	59	99.17	22	6992	11	8	0.73	0.67	0.99
Non-outbreak	1320	14186	72080944	7091	53	99.25	23	7001	8	7	0.77	0.74	0.99
Non-outbreak	1380	14471	73573256	7091	44	99.38	23	7008	10	7	0.77	0.70	0.99
Non-outbreak	1440	14683	74801565	7091	40	99.44	23	7012	10	7	0.77	0.70	0.99

The columns show (from left to right): (1) the sample analysed; (2) the cumulative results at this time period (min); (3) the total number of two-direction reads; (4) the total number of nucleotide bases; (5) the total size of the alignment; (6) the number of bases in the alignment missing from the dataset; (7) the percentage of bases in the alignment that can be called; (8) the count of true positives; (9) the count of true negatives; (10) the count of false positives; (11) the count of false negatives; (12) the recall, that is, sensitivity, calculated as TP/(TP + FN); (13) the precision, calculated as TP/(TP + FP); (14) the accuracy, calculated as (TP + TN)/(P + N)

DNA strands as they move through a protein nanopore. A unique property of this technology is that sequence data are available in real time, and analysis can be performed on a continuous stream of long reads. We wished to evaluate the potential impact of a real-time approach for analysis of clinical bacterial isolates. We exploited this feature to perform rapid identification and typing of genomic DNA prepared from a pure colony isolate. Given the high error rate reads generated in this study we employed a database of taxon-defining genes from microbial species to make bacterial and bacteriophage identifications [15]. This approach is tolerant of low-coverage, high-error reads making it useful for real-time analysis of nanopore sequences. However, due to the higher error rate of this platform, a *de novo* SNP calling approach as utilised with MiSeq data would not produce informative results within the short time scales of interest here. Other studies have investigated the error rate and mode of this instrument in greater detail [15, 16, 17]. We show that despite the high error rate, effective genotyping is possible using phylogenetic placement techniques. Phylogenetic placement has been used to good effect in metagenomics studies where only low-coverage data are available, for example in the diagnosis of infectious diseases from ancient DNA samples, directly from sputum and from the hospital environment [18–20]. Using this approach, and a simple heuristic algorithm to call the most likely genotype it was possible to reliably place streaming nanopore data onto a reference phylogeny despite the high read error rate. Other studies have shown that genotyping accuracy can reach 99 % when very high coverage (>120×) is available. This would permit a *de novo* genotyping approach which did not rely on phylogenetic placement, as is more typical in studies employing traditional high-throughput sequencing [14].

Both the draft sequencing protocol presented for the MiSeq and the real-time evaluation of nanopore sequencing demonstrate that these approaches have utility for generating data of use in outbreak investigations in less than one day (Fig. 6). It is not our intention here to perform direct comparisons between the instruments in this study, particularly as they are quite different in their mode of operation.

The MiSeq is typically run in a factory-style 'batch' mode, where many bacterial samples (up to 100 on a MiSeq, or potentially many hundreds on the larger HiSeq instrument) are run simultaneously, and processed in



Serovar	Strain
Gallinarum	287/91
Gallinarum	9
Pullorum	S06004
Gallinarum/Pullorum	CDC1983-67
Pullorum	ATCC 9120
Enteritidis	58-6482
Enteritidis	436
Enteritidis	6.0562-1
Enteritidis	13-Jan
Enteritidis	629164-37
Enteritidis	22704
Enteritidis	50-5646
Enteritidis	653049
Enteritidis	607308-16
Enteritidis	648904

Fig. 4 (See legend on next page.)

(See figure on previous page.)
Fig. 4 Results of streaming phylogenetic placement from the Oxford Nanopore MinION on a reference tree of representative published *Salmonella enterica* sequences. Common serovars of Salmonella are highlighted. Both outbreak and non-outbreak strains are unambiguously identified as *Salmonella enterica* serovar Enteritidis by their position on the phylogenetic tree within 50 min. The line demonstrates the potential range of placements reported by *pplacer*. The red placements indicate the positions of the outbreak isolate and the blue placements indicate the positions of the non-outbreak isolate

serial at the end of the instrument operation. This approach reduces the cost of sequencing by taking advantage of the very high output offered from these instruments (>1 terabase for the HiSeq in High Output Mode). The precipitous drop in the cost of sequencing bases has meant that for bacterial applications the cost of library preparation is rapidly becoming the most expensive component. However, batch methods, particularly with the very highest output modes result in a flexibility trade-off; such an approach means that data cannot be analysed until at least the barcode identifiers have been read (usually not until after halfway through the run).

This is in contrast to the real-time sequencing approach of the MinION whereby individual samples are loaded, and results are generated and analysed in real time until the results are sufficient to address the clinical question. Such an approach has appealing properties for applications such as infectious disease diagnostics. A second attribute of the MinION that is notable is its extreme portability, comparable in size to a USB flash drive and requiring only a basic laptop to draw power from and connect to. This suggests that it may, in principle, be possible in the future to move sequencing closer to the sample, and particularly when coupled with a culture-independent approach.

However, at present the instrument depends on access to a basic molecular biology laboratory infrastructure, including access to freezer, and basic laboratory equipment such as heater blocks and pipettes. The existing library preparation method, although relatively quick, is quite labour-intensive for each sample. Presently there is no method for multiplexing large numbers of bacterial genomes (as with the MiSeq instrument), nor would the throughput be amenable to this. Therefore, it seems likely for large-scale surveillance efforts this platform is not the obvious choice, for reasons of labour and cost. Instead, we envisage that development of rapid library preparation assays will be necessary in order to see this platform become usable in a clinical microbiology laboratory or patient setting in the manner described here.

Furthermore, the need for culture enrichment remains a significant bottleneck for rapid identification of bacteria and this also applies to other studies employing whole-genome sequencing. Culturing of *Salmonella* takes between 24 h (presumptive diagnosis) and 48 h (pure culture for sequencing). Our approach, which relies on sequencing single colonies from each sample, is a limitation of this and similar studies. However, sequencing of four individual colonies from the food trolley demonstrated very limited heterogeneity with three isolates being identical to the majority of other cases in the outbreak, and one showing two SNP differences. A culture-free approach for bacterial diagnostics has been recently proposed and this would permit detection of mixed infections as well as cutting down the time to result significantly, for example in the case of direct sequencing of Shiga-toxin producing *E. coli* from stool samples and *M. tuberculosis* directly from sputum [21, 22]. However, sequencing mixed communities reduces the genomic coverage of the pathogenic target of interest, and so for such an approach to be successful it is likely to rely on generating greater throughput than currently achievable on the MinION. Enrichment for the target organism, most easily attained through traditional microbiology culture, is therefore still a required stage.

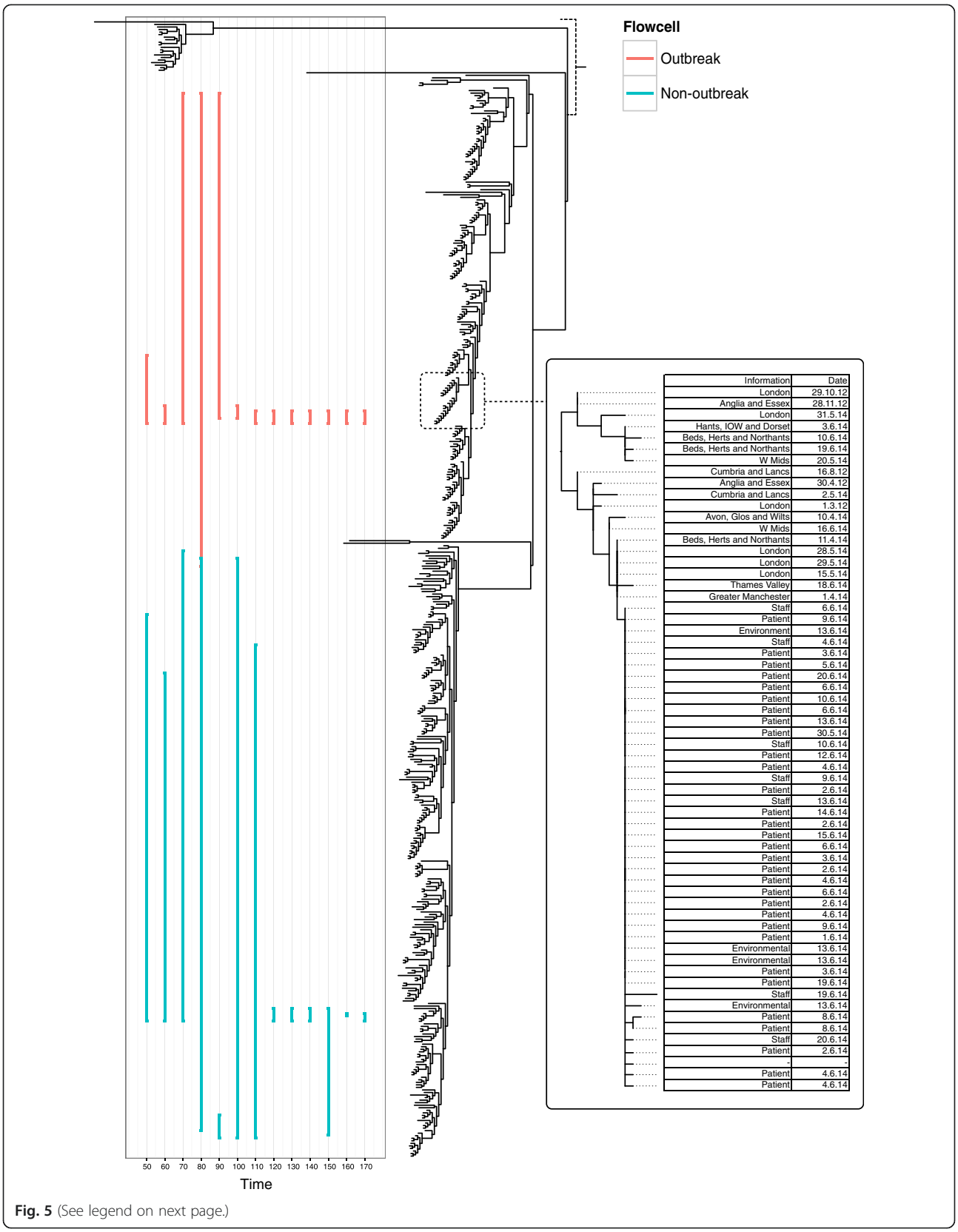
Conclusion

The combination of rapid prospective sequencing during an outbreak and detailed characterisation of cases occurring on a national scale has potential implications for the future of outbreak investigation [23]. We describe a novel protocol for draft sequencing on the MiSeq that is sufficiently quick to determine whether an outbreak is occurring. For this vision to become a reality, further work is needed to enable sharing of data between hospitals and community practitioners with public health laboratories. Larger scale integration with national genome databases represents the first implementation of a new paradigm for the investigation of outbreaks. The use of rapid, draft sequencing can delineate the context of an outbreak very quickly even at lower than usual genome coverage.

Materials and methods

Sample and bacterial culture collection

Faeces samples from patients with diarrhoea were submitted for culture and plated on XLD medium. Presumptive *S. enterica* isolates were confirmed using biochemical tests and O- and H-antigen agglutination sera and all those identified as *S.* Enteritidis were retained for molecular typing. Environmental swabs were taken from the affected wards within 24 h of the ward clusters being identified and were processed as above.



Information	Date
London	29.10.12
Anglia and Essex	28.11.12
London	31.5.14
Hants, IOW and Dorset	3.6.14
Beds, Herts and Northants	10.6.14
Beds, Herts and Northants	19.6.14
W Mids	20.5.14
Cumbria and Lancs	16.8.12
Anglia and Essex	30.4.12
Cumbria and Lancs	2.5.14
London	1.3.12
Avon, Glos and Wilts	10.4.14
W Mids	16.6.14
Beds, Herts and Northants	11.4.14
London	28.5.14
London	29.5.14
London	15.5.14
Thames Valley	18.6.14
Greater Manchester	1.4.14
Staff	6.6.14
Patient	9.6.14
Environment	13.6.14
Staff	4.6.14
Patient	3.6.14
Patient	5.6.14
Patient	20.6.14
Patient	6.6.14
Patient	10.6.14
Patient	6.6.14
Patient	13.6.14
Patient	30.5.14
Staff	10.6.14
Patient	12.6.14
Patient	4.6.14
Staff	9.6.14
Patient	2.6.14
Staff	13.6.14
Patient	14.6.14
Patient	2.6.14
Patient	15.6.14
Patient	6.6.14
Patient	3.6.14
Patient	2.6.14
Patient	4.6.14
Patient	6.6.14
Patient	2.6.14
Patient	4.6.14
Patient	9.6.14
Patient	1.6.14
Environmental	13.6.14
Environmental	13.6.14
Patient	3.6.14
Patient	19.6.14
Staff	19.6.14
Environmental	13.6.14
Patient	8.6.14
Patient	8.6.14
Staff	20.6.14
Patient	2.6.14
-	-
Patient	4.6.14
Patient	4.6.14

Fig. 5 (See legend on next page.)

(See figure on previous page.)
Fig. 5 Results of streaming phylogenetic placement from the Oxford Nanopore MinION on a reference tree of *Salmonella enterica* serovar Enteritidis isolates collected by Public Health England during routine surveillance. The left-most panel demonstrates the confident placing of the outbreak isolate in the outbreak clade within 100 min, and the confident placing of the non-outbreak isolate into a clade containing multiple serotypes of *Salmonella* within 120 min. The red placements indicate the positions of the outbreak isolate and the blue placements indicate the positions of the non-outbreak isolate. The right-most panel shows a phylogenetic reconstruction of isolates from the outbreak and their source, set in context of a national outbreak of phage type 14b. Uncertainty in the phylogenetic placement technique is demonstrated early on in sequence data collection due to the low accuracy of the variant calls collected. As more data are collected, the number of possible phylogenetic placements reduces and the confidence values increase (not shown)

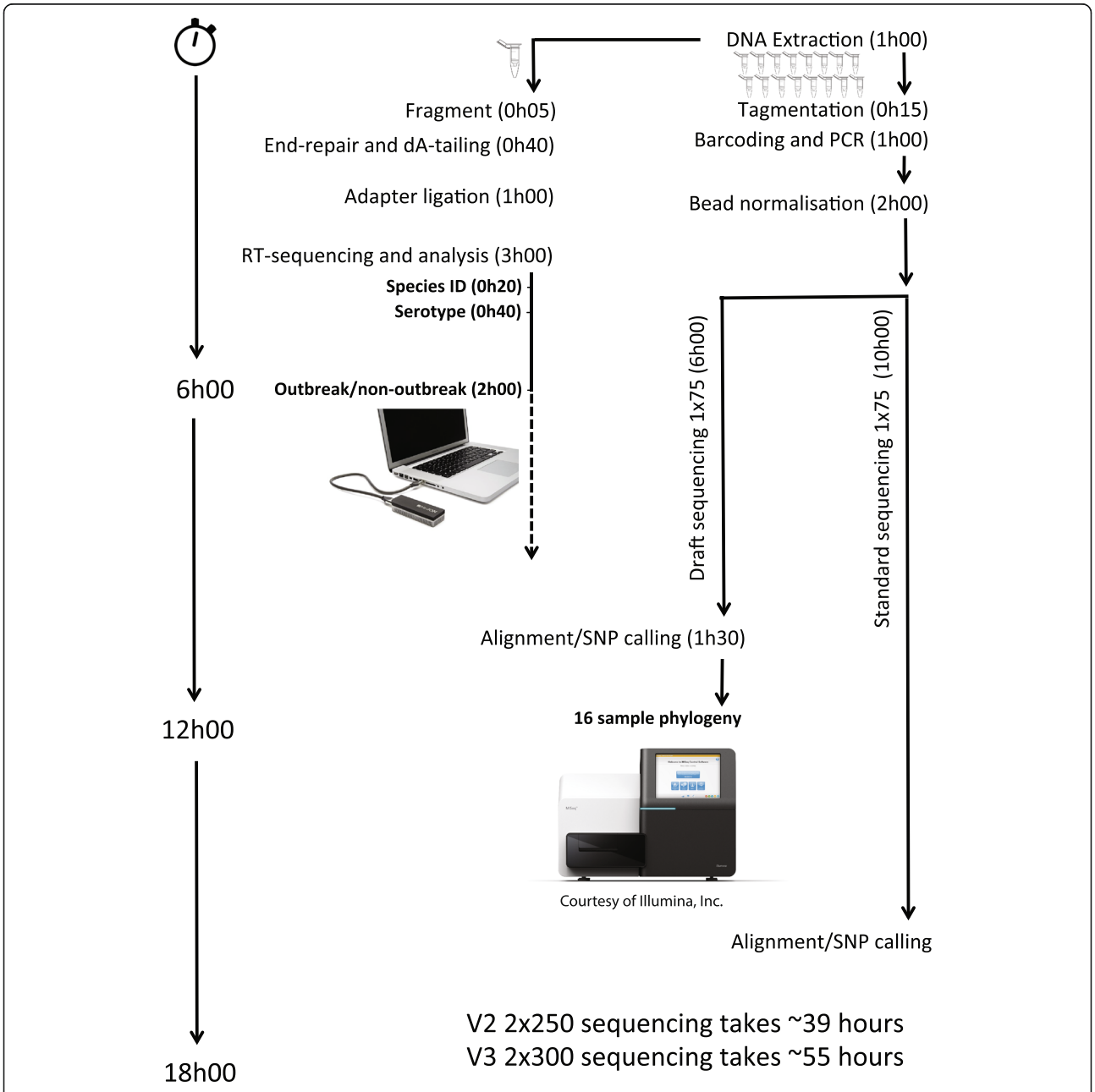


Fig. 6 A schematic showing time-to-result for the sequencing analysis strategies used in this study, starting from DNA input

Genomic DNA extraction

Genomic DNA was prepared from nutrient agar slopes incubated for 4–18 h at 37 °C. Cells were harvested using 100 μL of sterile PBS added to the surface and a sterile loop used to emulsify bacteria into a suspension that was then pipetted into a sterile Eppendorf tube. This suspension was used to harvest DNA with the 'Invisorb spin cell mini kit' (Invitek, Germany) according to the manufacturer's instructions. The quantity of DNA in each sample was determined using a Qubit 2.0 fluorometer and dsDNA HS assay (Life Technologies, Paisley, UK).

Library preparation for Illumina MiSeq sequencing

Sequence-ready libraries were generated from 1 ng DNA per sample using the Nextera XT library preparation kit (Illumina, Great Chesterford, UK) according to the manufacturer's instructions.

Rapid draft sequencing on the Illumina MiSeq

In order to provide results for an emergency infection control meeting the next morning, we adapted the standard sequencing protocol on the Illumina MiSeq to rapidly generate sufficient data to analyse 16 strains. We utilised a standard *V3* 600-cycle reagent kit. By modifying the recipe files on the instrument we reduced the chemistry time by 40 s per cycle and the number of tiles imaged by 50 %. This resulted in a cycle time of approximately 3 min per cycle and allowed 75 base single-read sequencing with dual barcoding to complete within 6 h. We chose 75 base reads as a trade-off between expected genome coverage and available time in order to have results available sufficiently quickly for analysis. The sequencing protocol can be downloaded from [24].

Standard sequencing on Illumina MiSeq and HiSeq

Later in the outbreak isolates were sequenced using the Illumina MiSeq with standard V3 protocol at the University of Birmingham, UK, also prepared with Nextera XT reagents. In addition, some outbreak isolates were sequenced on the Illumina HiSeq 2500 with TruSeq V3 reagents as part of the Public Health England (PHE) WGS sequencing pipeline at Colindale, UK (Additional file 1: Table S1).

Phylogenetic reconstruction from draft sequencing

Before being mapped against the reference genome *S.* Enteritidis P125109 (PRJNA59247) with BWA-MEM (version 0.7.5), 75 base single-read data generated by draft sequencing on the Illumina MiSeq was adapter and quality trimmed with Trimmomatic [25, 26]. Single nucleotide polymorphisms (SNPs) were called using samtools mpileup (version 0.1.18) and VarScan (version 2.3.6), specifying a minimum read depth of 2 [27, 28]. Filtered SNPs (those positions with an allele frequency of >80 % to call a variant or <20 % to call the reference base in all samples) were extracted to make a concatenated FASTA alignment. FastTree (version 2.1.7) was used to generate an approximate maximum likelihood phylogenetic tree [29]. Phyloviz was used to produce minimum spanning tree reconstructions [30]. Functional annotation of these variants was performed using snpEff (version 3.1) [31].

Phylogenetic reconstruction from PHE surveillance sequencing

Before being mapped against the reference genome *S.* Enteritidis AM933172 (PRJEA30687) with BWA-MEM, 100 base pair paired-end data generated on the Illumina HiSeq 2500 was adapter and quality trimmed [26]. SNPs were called using GATK [32]. High quality SNPs (>10-fold coverage, >30 mapping quality, 90 % consensus) were selected and uploaded into SNPdatabase (SNPdb). This is an in-house PostgreSQL database containing genome position and variant base for each SNP and low quality/missing positions for all *S.* Enteritidis eBURST group 4 (EBG 4) isolates sequenced by PHE. SNPs in the core genome of the strain set being analysed were extracted from an in-house SNPdb and FastTree was used to derive approximate maximum likelihood phylogenetic trees. Annotation data came from the in-house PHE GastroDataWarehouse (GDW).

Due to the clinical interest in these cases, strains with below standard sequencing depth (30×) were analysed and this had no impact on the analysis outcome, with identical tree topologies detected in all cases.

Real-time sequencing on the MinION

An outbreak and a non-outbreak isolate, as determined by earlier MiSeq sequencing, were chosen for sequencing on the MinION (Oxford Nanopore Technologies, Oxford, UK) to assess its suitability for future outbreak investigations. High-molecular weight input DNA (1 μg) was fragmented using a Covaris G-Tube (Covaris, Woburn, USA) at 5,000 rpm in a centrifuge. Fragmented DNA was end-repaired using the NEB repair module (New England Biolabs, Ipswich, USA), then cleaned-up using SPRI beads with a ratio of 1:1 beads to reaction mixture. End-repaired DNA was then A-tailed using the NEB A-tailing module. Following this a sequence-ready library was generated using the gDNA sequencing kit and protocol provided as part of the MinION access program (MAP). The diluted library (150 μL) was loaded into the MinION flowcell via the sample loading port. A 72-h sequencing protocol was initiated using the MinION control software, MinKNOW (version 0.45.2.6). Read event data were base-called by the software Metrichor (version 0.16.37960) using workflow 1.0.2_R7. The

FASTA sequences and strand translocation times were extracted for further analysis using the poretools FASTA extraction function [33]. All sequence data are deposited into the Short Read Archive (SRA) with study reference ERP006904 (MinION data) and ERP007194 (Illumina data).

Species identification from nanopore reads

Identification of bacterial and viral species present in each sample was carried out using an alignment method to the MetaPhlAn 2 database of taxon-defining marker genes [33]. First, the database was extracted into FASTA format using the fastacmd utility supplied with NCBI BLAST. Alignment of nanopore reads was performed using the LAST package (version 475), invoking lastal with custom settings as per Quick *et al.* [15], using a gap creation penalty and extension of 1 and a mismatch penalty of 2 (match score 1), corresponding to command line arguments -a1 -b1 -q2.

Subspecies level classification from nanopore reads

Serovars of *S. enterica* can often be assigned by phylogenetic methods. A phylogenetic reference tree was created from the available draft or complete *Salmonella enterica* genomes in RefSeq. From each of the sequences 600,000 simulated paired-end reads were generated using wgsim (version 0.3.1) [34]. These were mapped against the reference genome *S.* Typhimurium LT2 (PRJNA57799) with BWA-MEM (version 0.7.5) [26]. samtools mpileup (version 0.1.18) and VarScan (version 2.3.6) were used to call variants [27, 28]. Variant filtering was done using filter_non_discriminatory_variants.py [35] in order to remove non-discriminatory positions, as well as heterozygous positions and regions of putative recombination. Variant alleles for each sample were concatenated into a fasta file using vcf2phyloviz.py [36]. This file was de-duplicated using the mogrify command in seqmagick (version 0.6.0) to remove identical sequences which can affect placements. A phylogenetic reconstruction was created using FastTree (version 2.1.7) following a generalised time reversible model, after which taxtastic (version 0.5.1) was used to build the reference package [37].

To determine the subspecies level classification from the nanopore sequencing data, the reads were mapped against the reference genome with lastal with settings -a1 -b1 -q2. For each read, the highest scoring alignment was taken before being converted into BAM format using samtools. Using samtools mpileup and the script get_alleles_from_pileup.py the alignment was interrogated at all coordinates used for the reference tree. Aligned bases at these coordinates were counted and the dominant allele was used if at least two concordant bases were in the alignment. Alleles were concatenated into an alignment. Gap characters were used to represent uncertain positions not meeting the above criteria. The phylogenetic placement utility, pplacer, was used to place the sequence onto the reference tree producing a file containing the most likely position and logML probability for this placement. Placements with a likelihood value of greater than −500 were excluded [37]. This placement process was repeated for the read dataset available at each timepoint (10 min apart). New reads generated during each 10 min time interval were mapped to the reference, converted to a BAM file and merged with the BAM file generated at the previous time period.

S. enterica outbreak reconstruction

As with the subspecies level classification, phylogenetic placement can be used as a method for classifying samples in or out of an ongoing outbreak and in a national and international context. In order to do this, we leveraged the routine surveillance sequencing of *S. enterica* by PHE using 575 *S.* Enteritidis genomes of phage type 14b. Using the method described above a phylogenetic reference tree was created for these genomes (448 remained after de-duplication) before the nanopore sequences were placed onto the tree to predict whether or not they belonged to the outbreak cluster.

Additional file

Additional file 1: Table S1. Isolate identifiers sequenced in this study using rapid draft MiSeq sequencing, standard MiSeq sequencing and HiSeq sequencing during routine PHE surveillance.

Competing interests

NJL is a member of the MinION Access Programme (MAP) and has received free-of-charge reagents for nanopore sequencing presented in this study. JQ has received travel and accommodation expenses to speak at an Oxford Nanopore-organised symposium. NJL and JQ have ongoing research collaborations with Oxford Nanopore but do not receive financial compensation for this.

Authors' contributions

NJL, ER and PH conceived the study. CC, SG, JH, SN, K. Neal, K. Nye, TP, EdP, ER, KS, AC, TD and PH performed the microbiological and epidemiological investigation. MW performed DNA extractions. SC and JQ performed sequencing. JQ, PA, TD, ER, PH and NJL analysed data. NJL, TD, PH and MW contributed reagents. NJL, PH and JQ wrote the manuscript. All authors read and approved the final manuscript.

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4 Real-time, portable genome sequencing for Ebola surveillance

4.1 Author contributions

N.J.L., **J.Q.**, M.K.O'S., D.W., S.G., M.W.C. conceived the study. N.J.L., **J.Q.**, M.K.O'S., S.A.W., J.T., P.R., D.T. designed the lab in a suitcase and laboratory protocol and initial validation. **J.Q.**, S.D., L.C., J.A.B., R.K., L.E.K., and A.Ma. performed MinION sequencing. N.J.L., **J.Q.** and J.T.S. performed bioinformatics analysis and wrote software. N.J.L., **J.Q.**, S.D., E.S., P.F., L.C., A.Mi., N.M. and I.R. analysed the data. G.D., A.R., N.J.L., **J.Q.** and G.P. performed phylogenetic analysis. N.J.L., **J.Q.**, S.D., M.W.C., S.G., M.K.O'S., A.R., E.S., P.F., I.R., A.Mi., and L.C. wrote the manuscript.

4.2 Author contributions (additional detail)

J.Q. developed the amplicon sequencing strategy, the lab in a suitcase, generated the early field sequencing data in Guinea and the validation data at PHE. J.Q. supported the bioinformatics analysis. J.Q. drafted all figures and contributed to the writing of the manuscript.

4.3 Abstract

The Ebola virus outbreak was the largest on record and responsible for the deaths of more than 11,000 people. Genome sequencing has become an important tool in response to viral outbreaks as it can provide a high-resolution view of pathogen evolution. We developed a portable 'lab-in-a-suitcase' capable of generating Ebola virus genomes sequences in the field using the portable MinION sequencer. By performing the sequencing close to the samples we were able to reduce processing time to as little as 24 hours. In this way we could provide an up-to-date view of the outbreak to the Ebola national coordination team in Guinea over an 8-month period until the outbreak was declared over. This information could identify new cases as being part of known chains of transmissions which generally clustered geographically but occasionally were transmitted over large distances. Genome sequences were integrated in real-time with another group performing sequencing in neighbouring Sierra Leone allowing us to identify multiple cross-border transmission events. In total 142 genome sequences were generated and shared online at regular intervals through Github. The Ebola outbreak is the most highly sampled outbreak in history with over 5% of known cases sequenced and despite the magnitude of the tragedy we hope this can become a blueprint for future outbreak responses.

4.4 Published manuscript

LETTER

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Real-time, portable genome sequencing for Ebola surveillance

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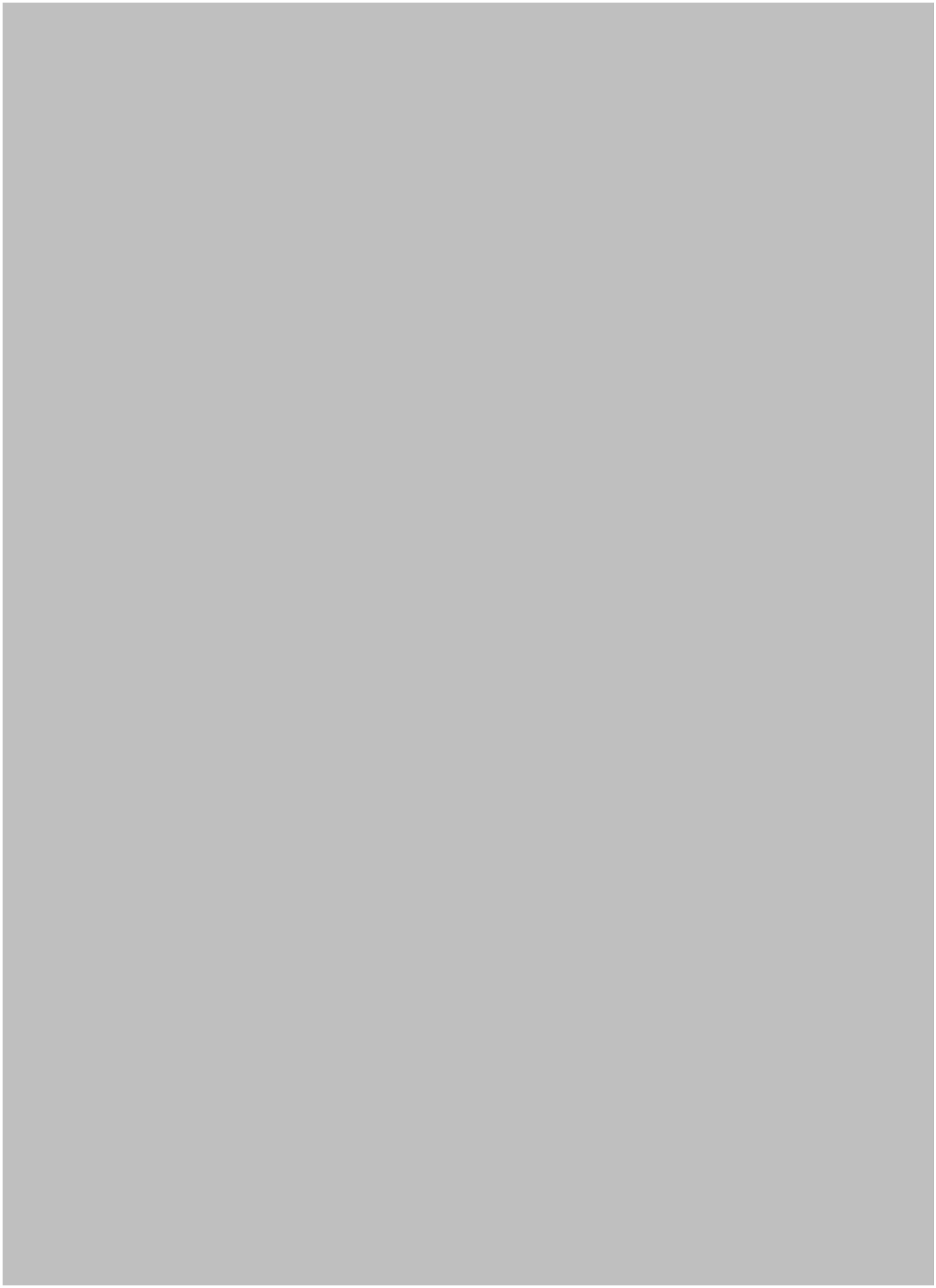
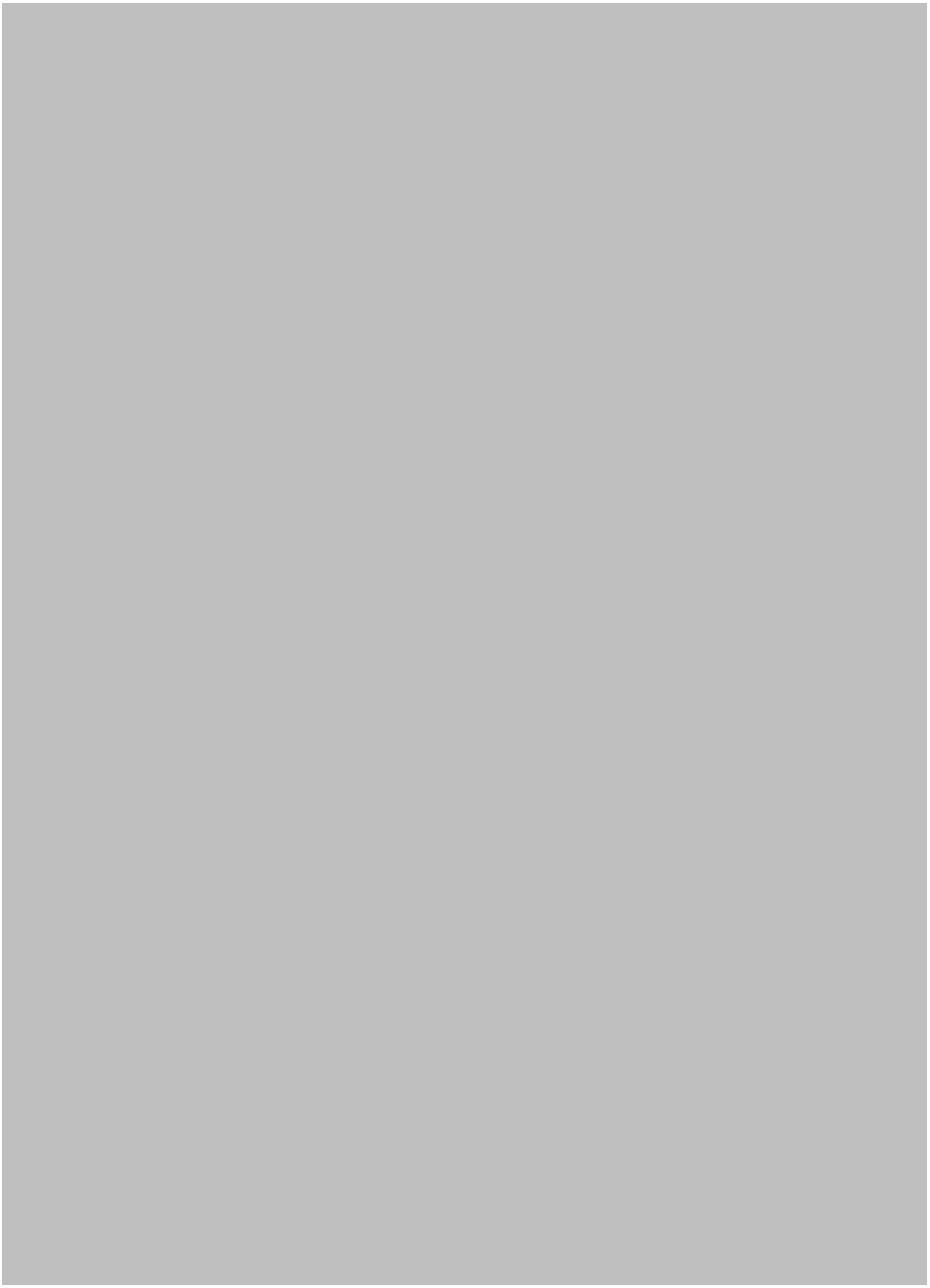
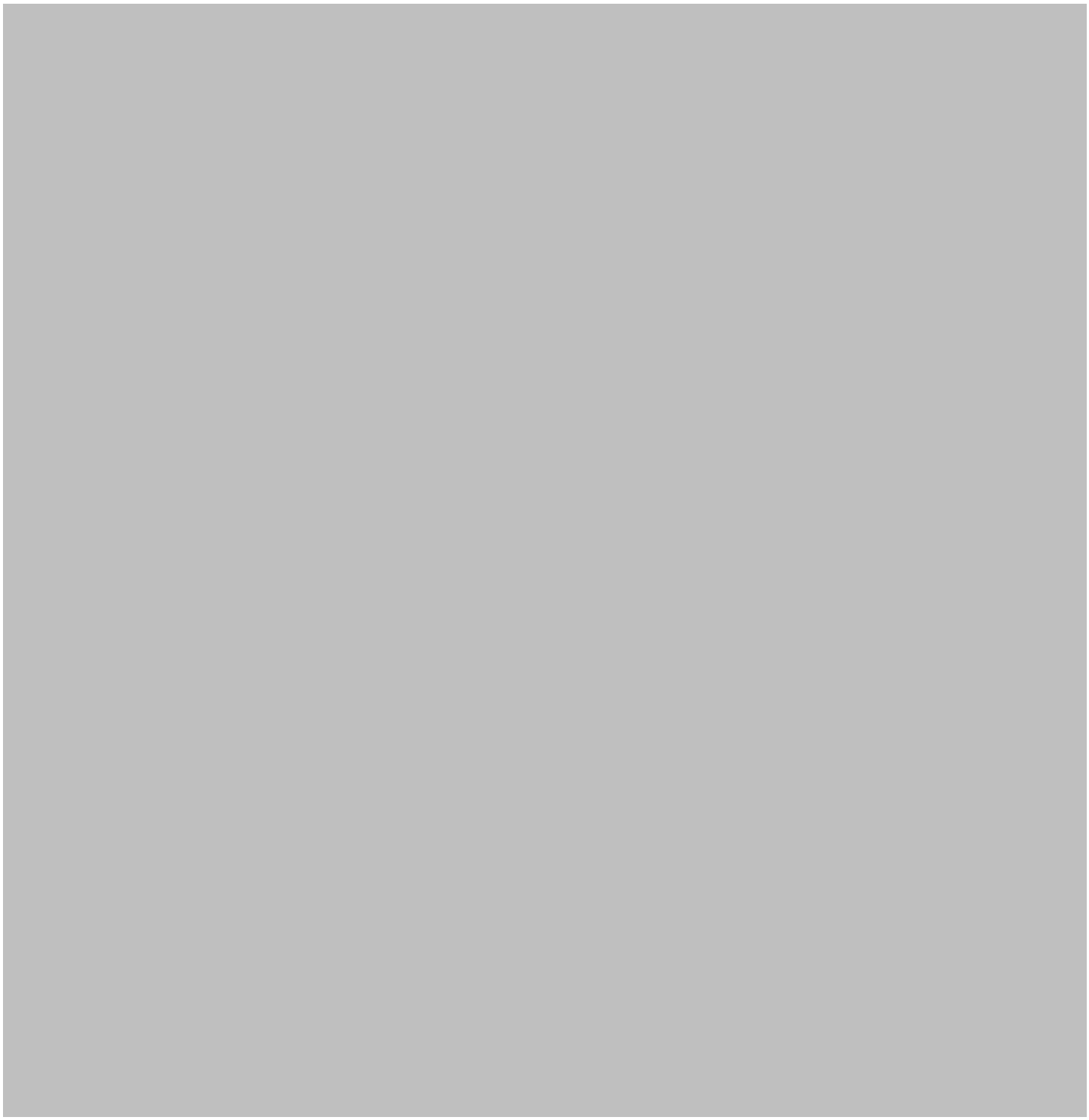
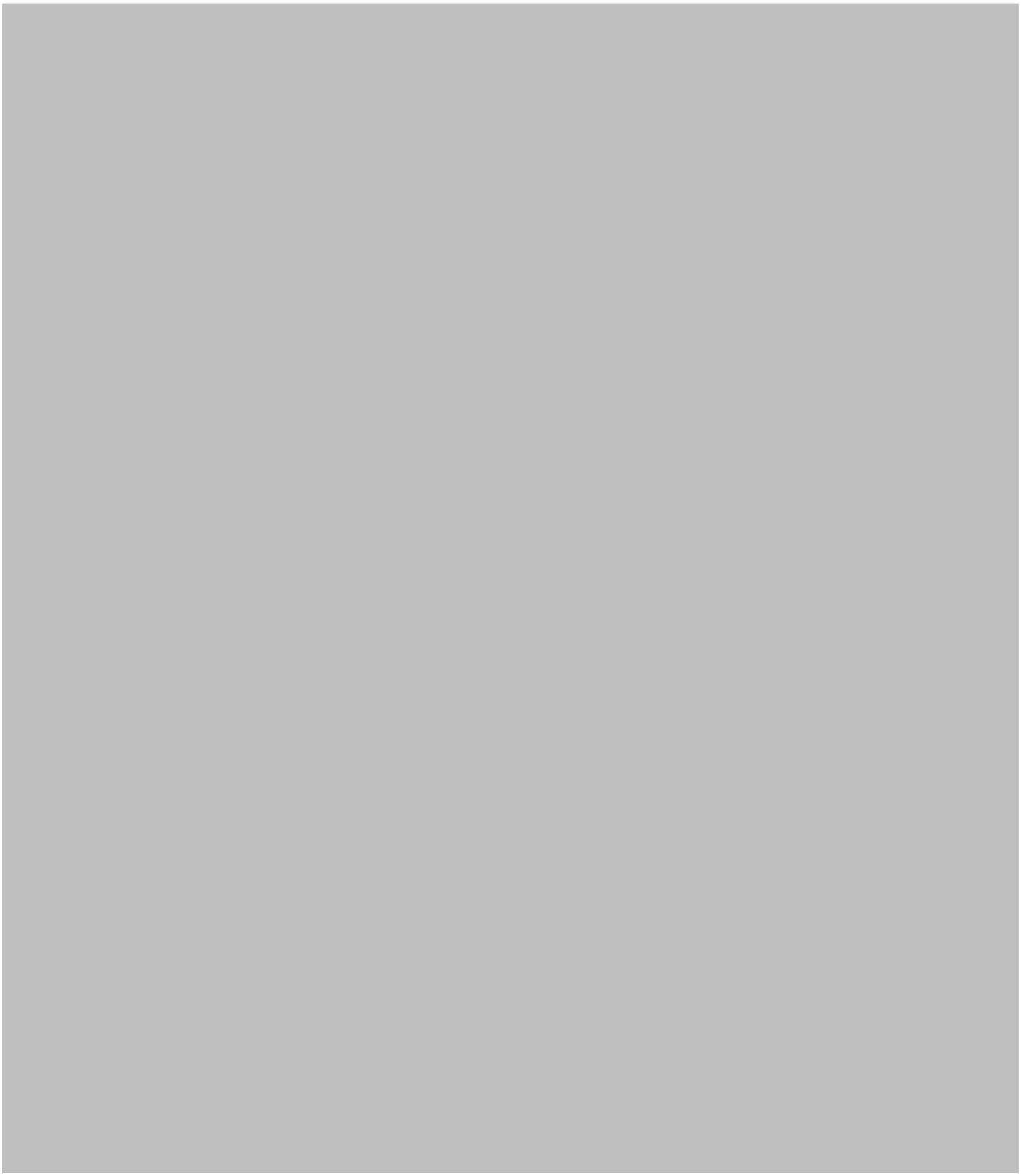
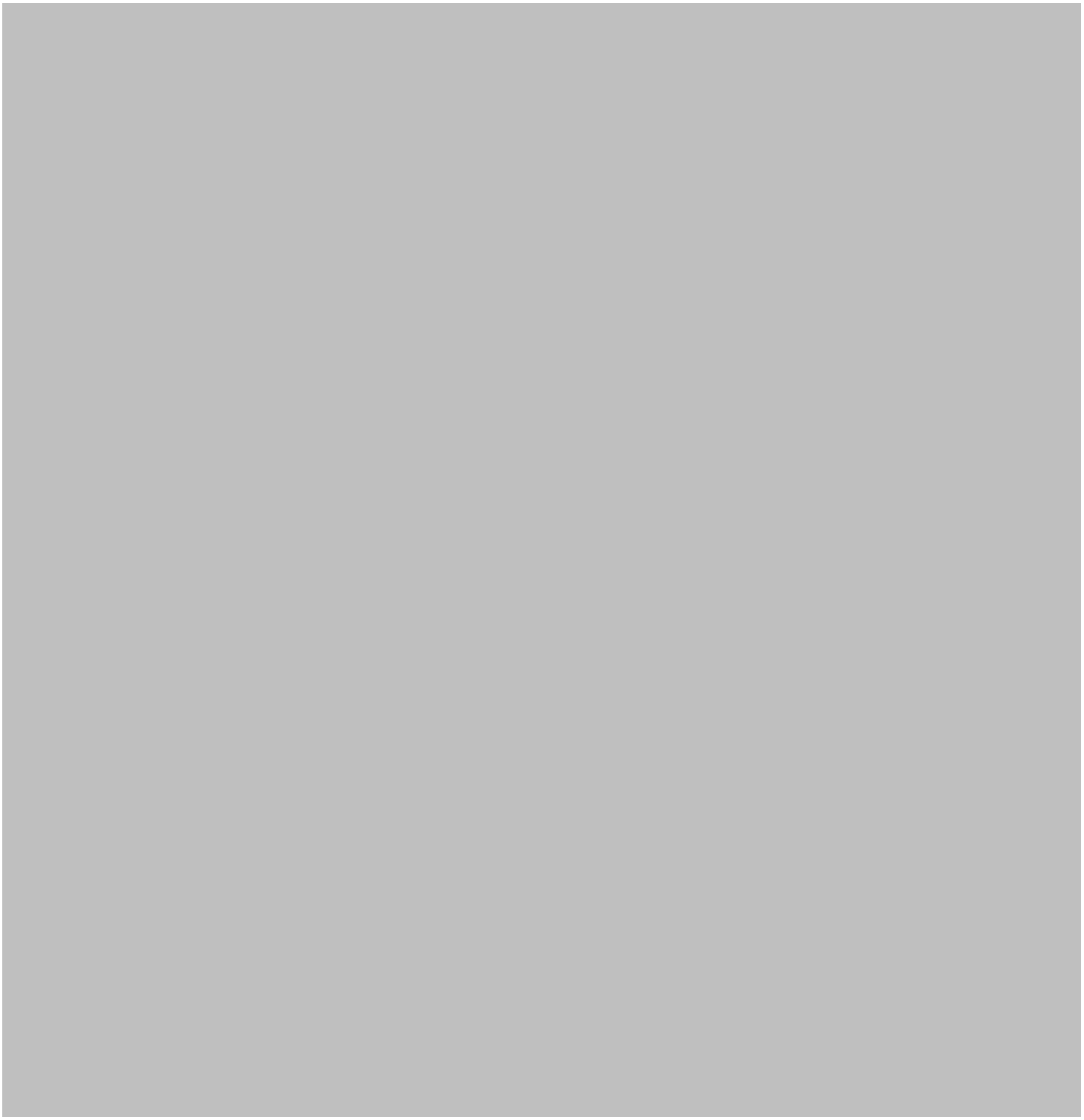
The Ebola virus disease epidemic in West Africa is the largest on record, responsible for over 28,599 cases and more than 11,299 deaths[1]. Genome sequencing in viral outbreaks is desirable to characterize the infectious agent and determine its evolutionary rate. Genome sequencing also allows the identification of signatures of host adaptation, identification and monitoring of diagnostic targets, and characterization of responses to vaccines and treatments. The Ebola virus (EBOV) genome substitution rate in the Makona strain has been estimated at between 0.87×10^{-3} and 1.42×10^{-3} mutations per site per year. This is equivalent to 16–27 mutations in each genome, meaning that sequences diverge rapidly enough to identify distinct sub-lineages during a prolonged epidemic[2–7]. Genome sequencing provides a high-resolution view of pathogen evolution and is increasingly sought after for outbreak surveillance. Sequence data may be used to guide control measures, but only if the results are generated quickly enough to inform interventions[8]. Genomic surveillance during the epidemic has been sporadic owing to a lack of local sequencing capacity coupled with practical difficulties transporting samples to remote sequencing facilities[9]. To address this problem, here we devise a genomic surveillance system that utilizes a novel nanopore DNA sequencing instrument. In April 2015 this system was transported in standard airline luggage to Guinea and used for real-time genomic surveillance of the ongoing epidemic. We present sequence data and analysis of 142 EBOV samples collected during the period March to October 2015. We were able to generate results less than 24 h after receiving an Ebola-positive sample, with the sequencing process taking as little as 15–60 min. We show that real-time genomic surveillance is possible in resource-limited settings and can be established rapidly to monitor outbreaks.

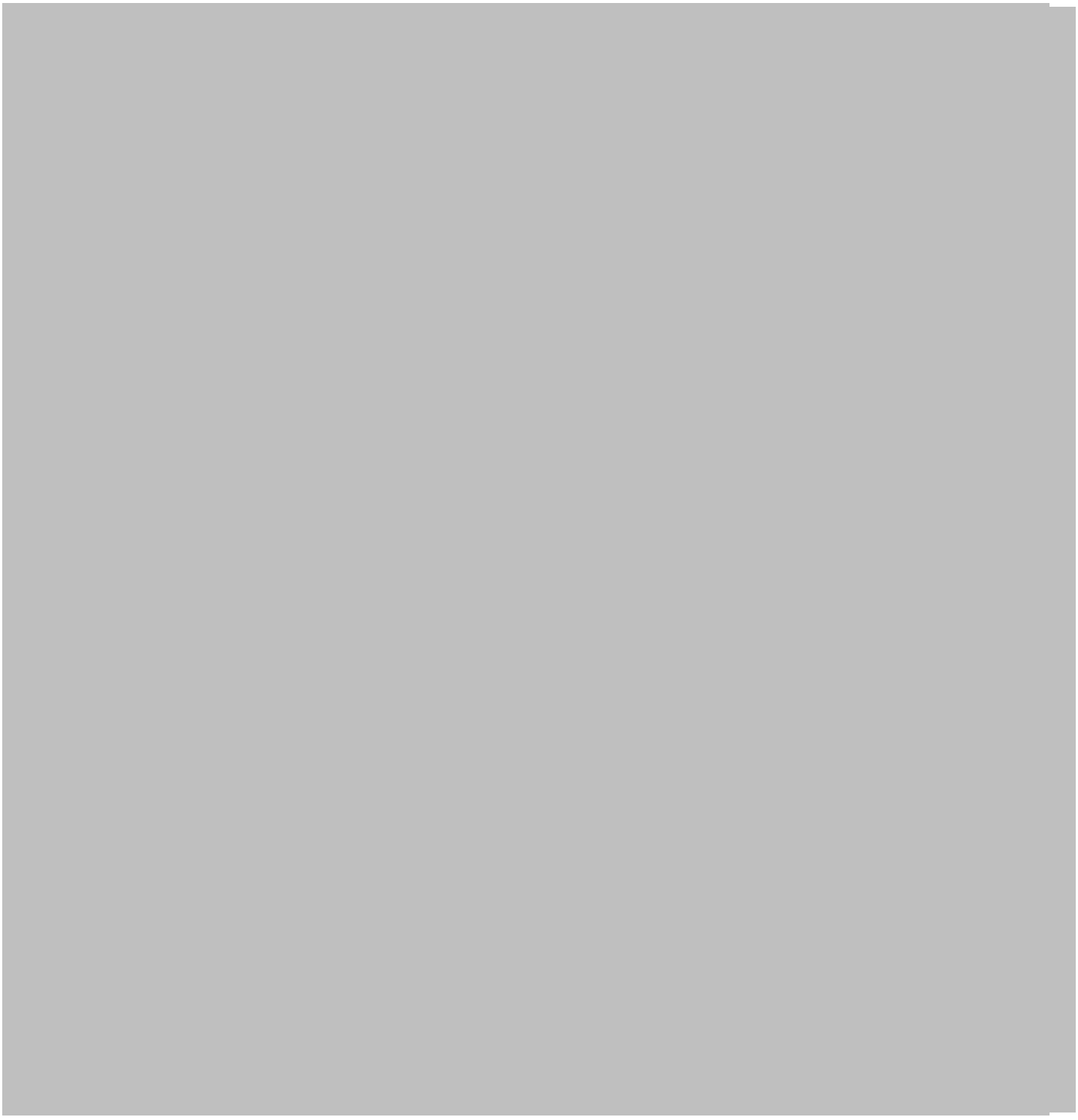


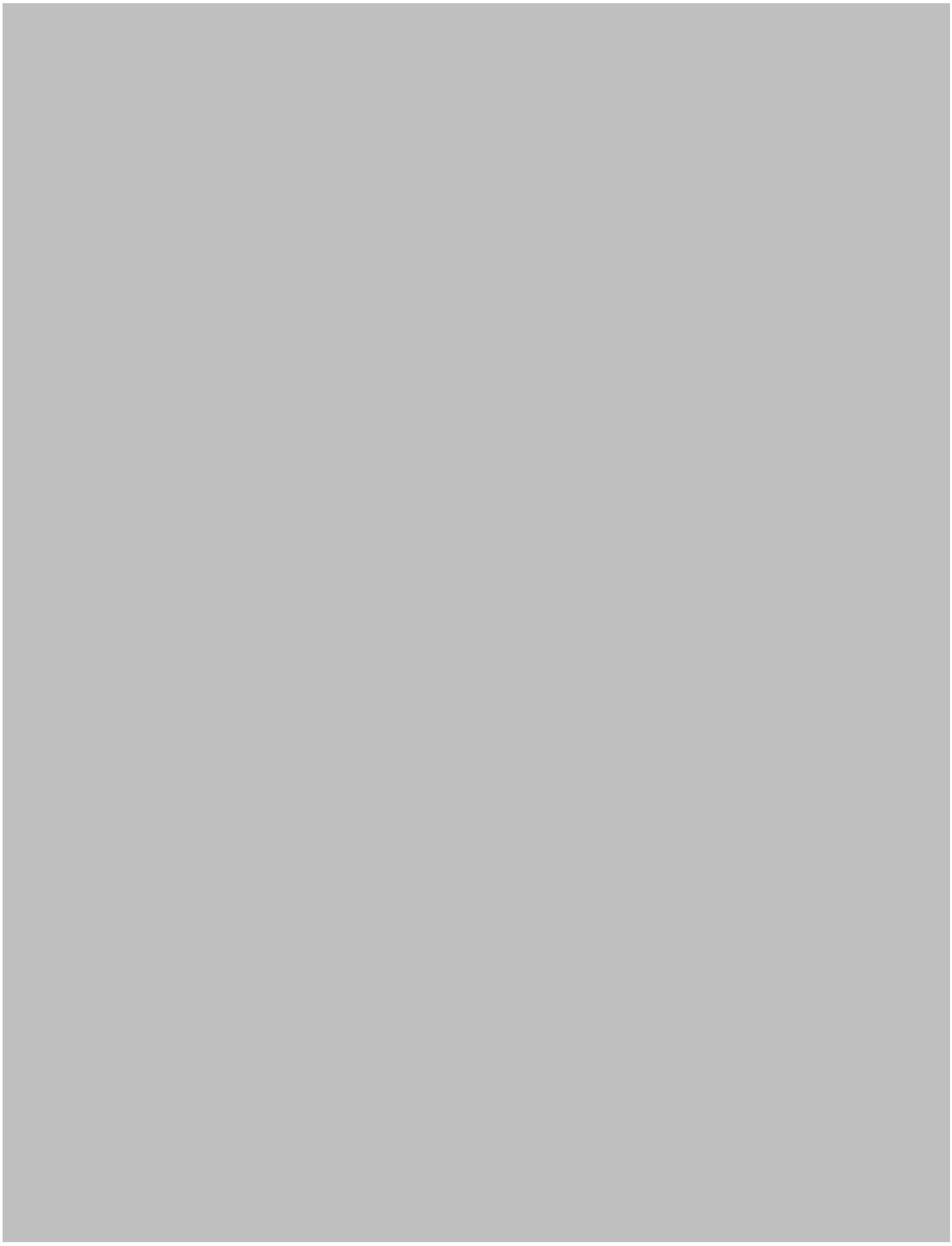


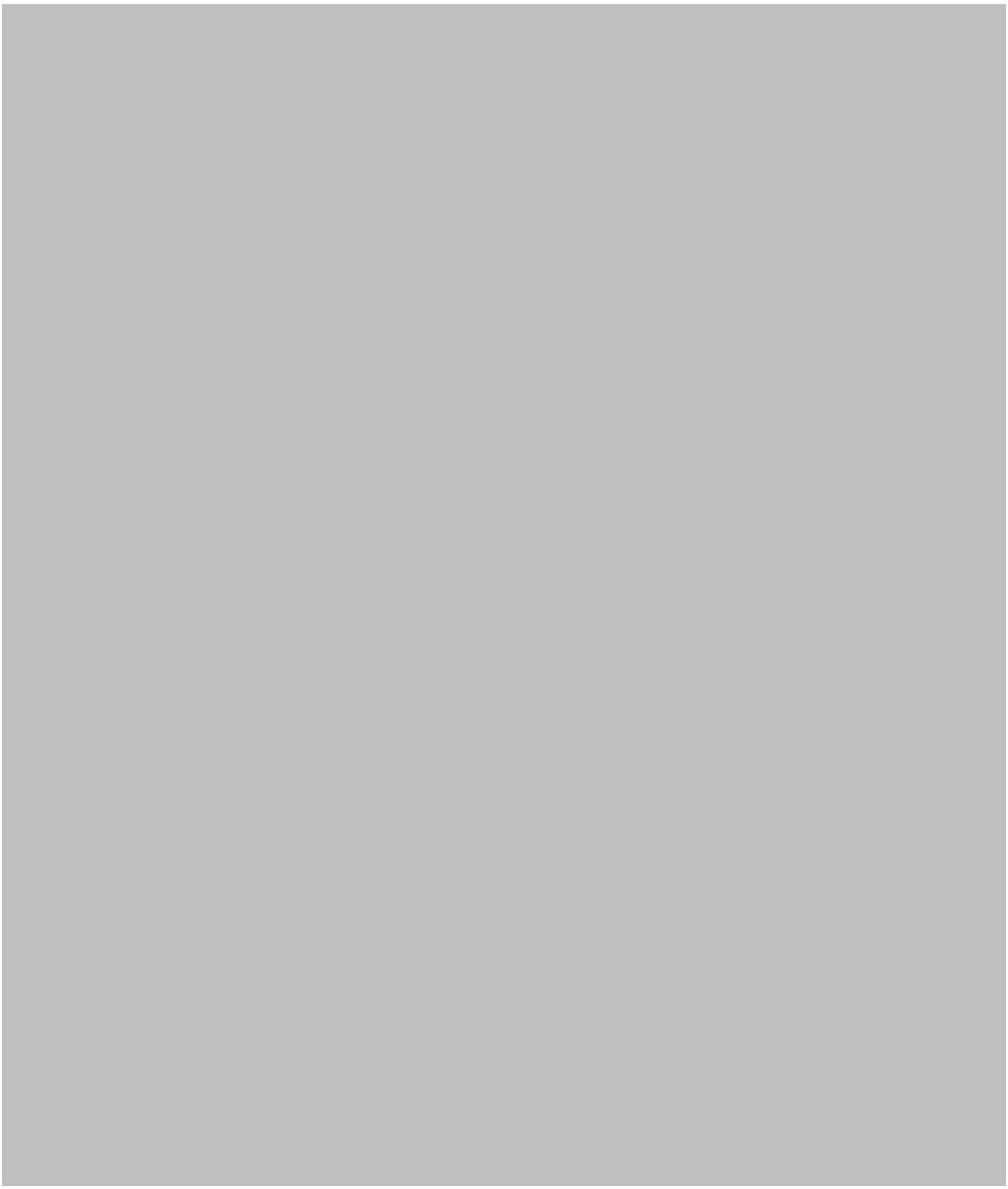


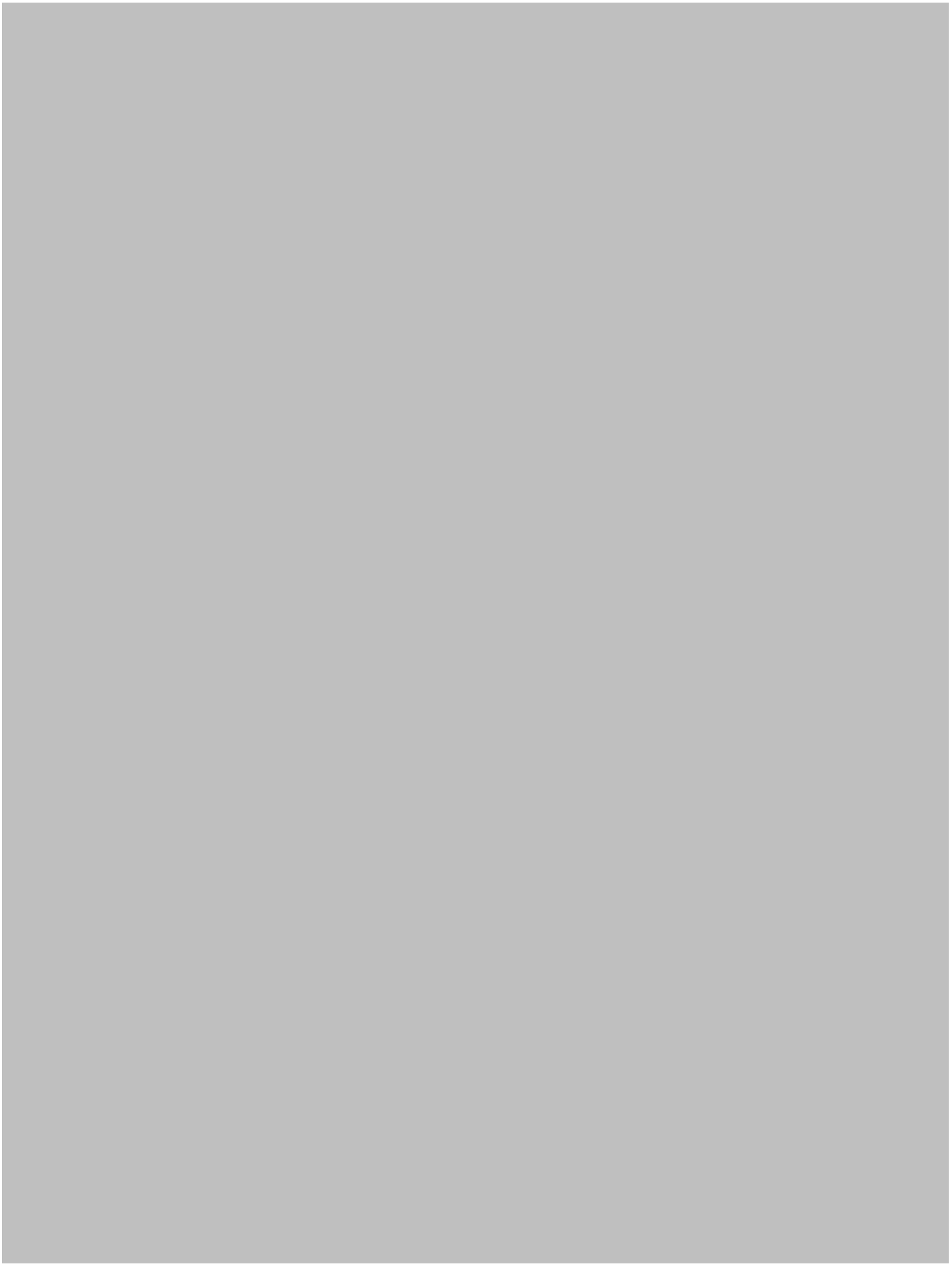


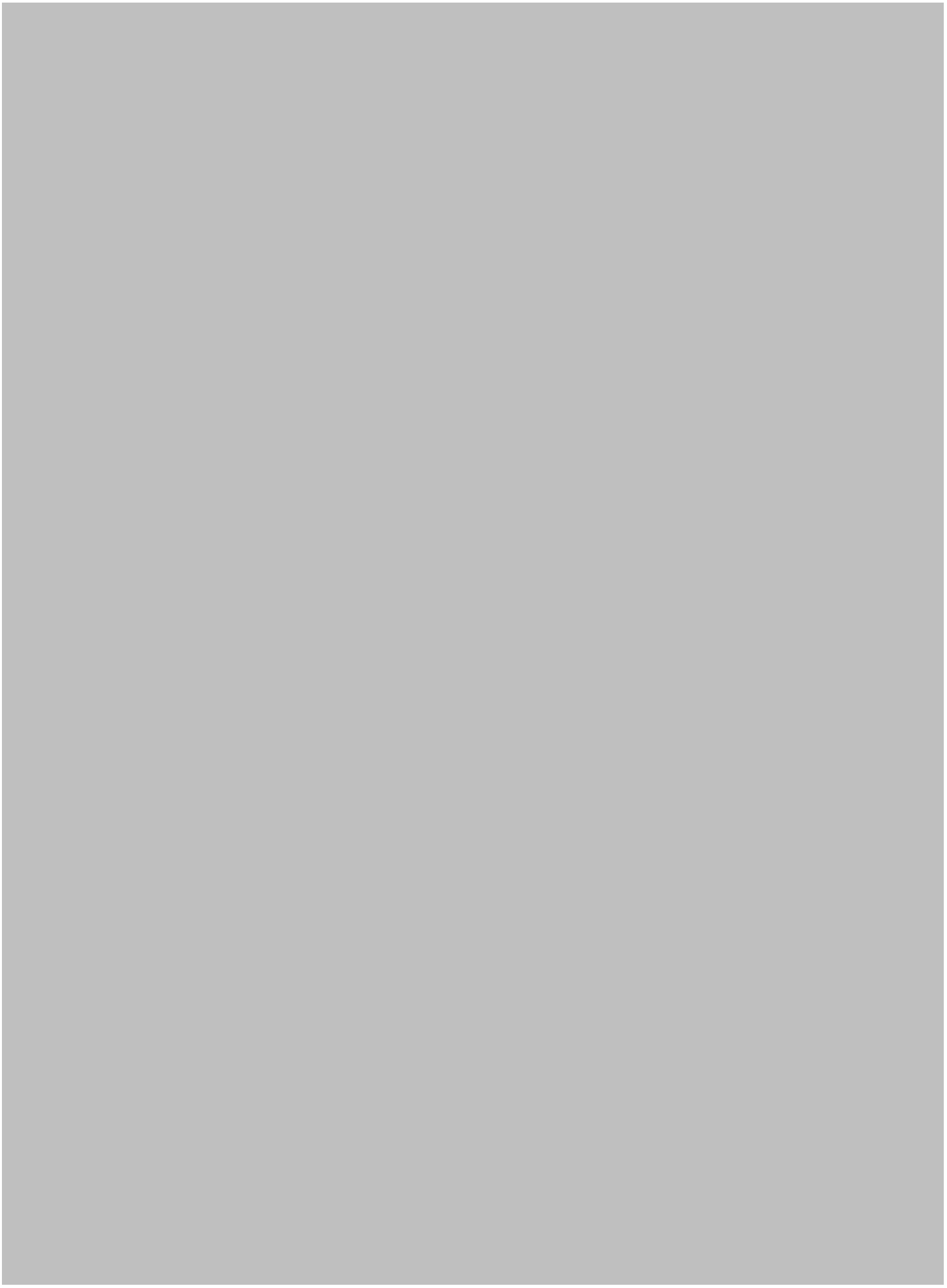


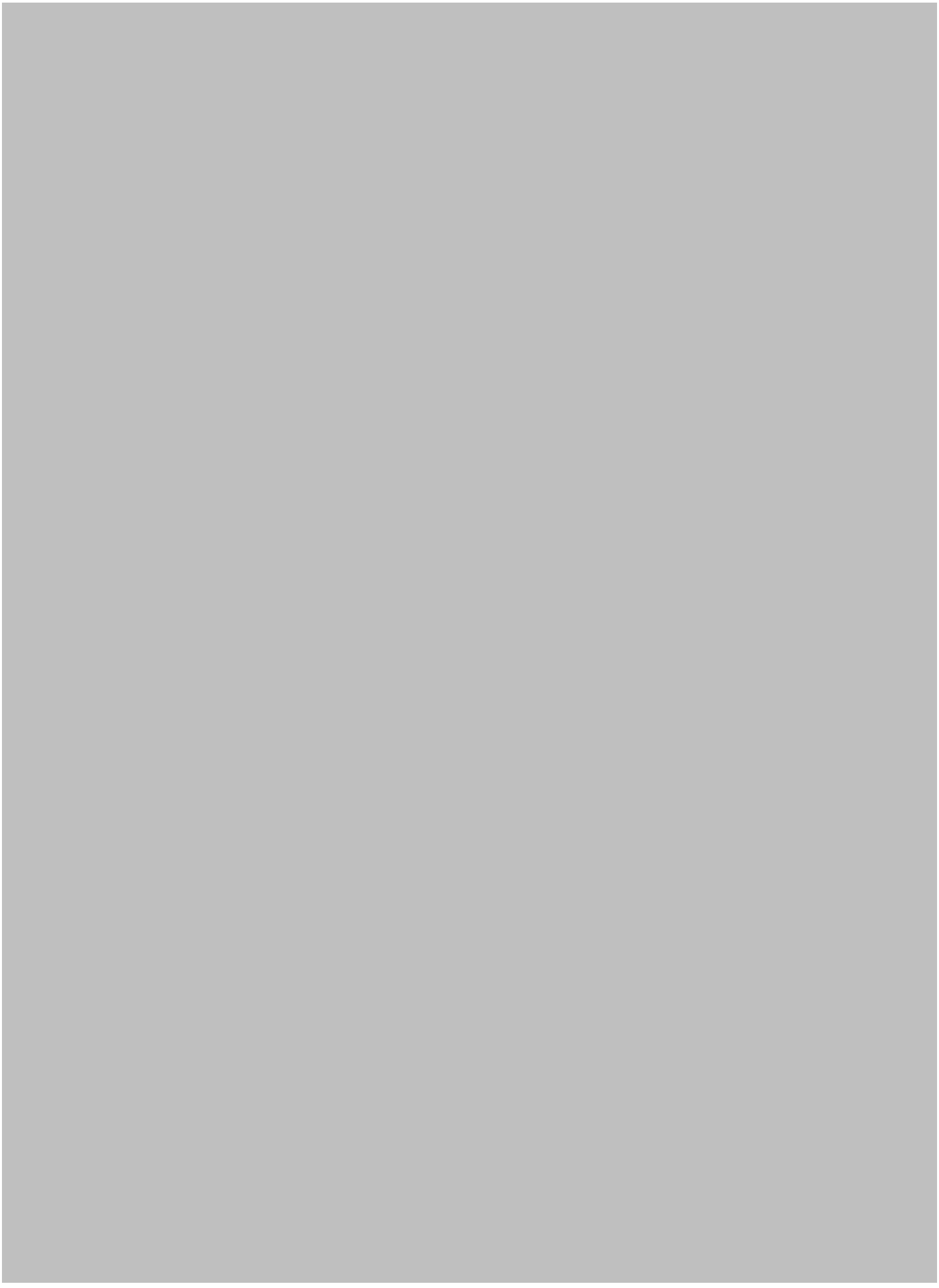


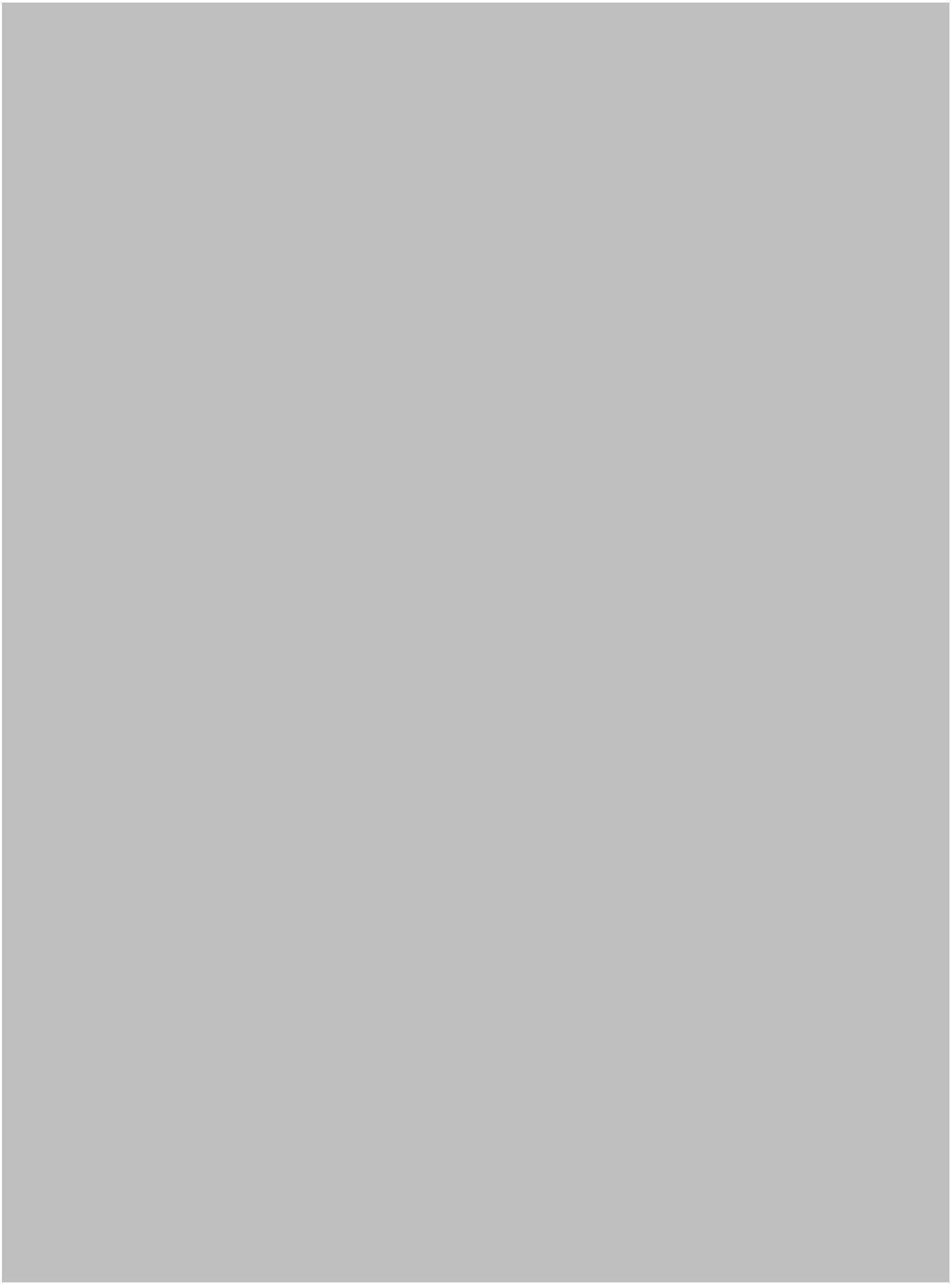


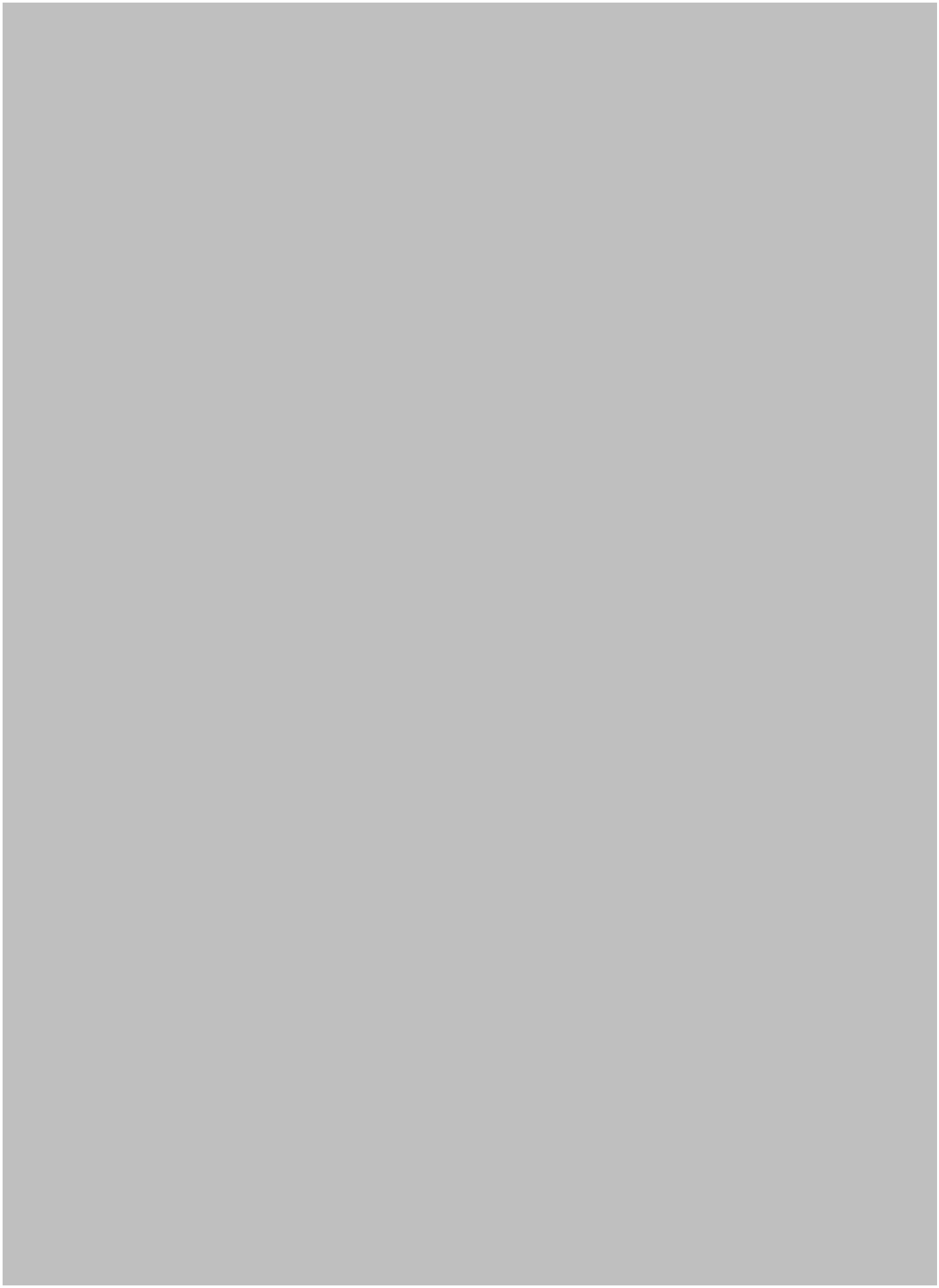












5 Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples

5.1 Author contributions

J.Q. and N.J.L. conceived the project. **J.Q.**, N.D.G., K.G.A. and N.J.L. designed the experiments and wrote the manuscript. **J.Q.**, A.D.S. and O.G.P. built the online primer design tool. All authors have read and approved the contents of the manuscript.

5.2 Author contributions (additional detail)

J.Q. developed the primer design tool, developed the laboratory protocol and performed the MinION validation experiments. J.Q. drafted the manuscript and all figures.

5.3 Abstract

Generating viral genomes from clinical samples can be challenging when the viral nucleic acid is present in very small amounts compared to a high background of host material. Zika virus infection produces a mild viremia and people often present to a physician after the peak viral load. The viral titre of typical Zika virus positive clinical samples is too low for metagenomic sequencing which when tested yielded small numbers of reads and incomplete genome coverage for a set of five clinical isolates. We instead developed a method for generation of many short overlapping amplicons in a

multiplex reaction. In order to simplify this process we built an online primer tool (primal.zibraproject.org) which automates the design procedure. We developed reaction conditions which could successfully generate amplicons covering most of the genome from real clinical samples up to Ct 36 representing very high sensitivity. We sequenced these amplicons using barcoding on the Oxford Nanopore MinION which reduced the cost to around $50 per sample when 12 samples were multiplexed on a single MinION flowcell. We produced a Docker container which enabling the generation of consensus sequences in a single pipeline which can be run on a laptop computer making it ideal for field situations. The general method is applicable to other viruses and we demonstrate the results of sequencing of Chikungunya virus reference material. Amplicon sequencing is a very sensitive method which makes it suitable for Zika virus sequencing however it has drawbacks including a reduction in efficiency when mismatches occur in primer binding sites, especially those close to the 3' end. This multiplex amplicon sequencing a useful tool in outbreak situations when strains are expected to be highly isogenic. It represents a robust and cost-effective method that can be performed in a day by someone who possesses basic molecular biology skills and we hope it will be useful method in future outbreaks.

5.4 Published manuscript

Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples

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Genome sequencing has become a powerful tool for studying emerging infectious diseases; however, genome sequencing directly from clinical samples (i.e., without isolation and culture) remains challenging for viruses such as Zika, for which metagenomic sequencing methods may generate insufficient numbers of viral reads. Here we present a protocol for generating coding-sequence-complete genomes, comprising an online primer design tool, a novel multiplex PCR enrichment protocol, optimized library preparation methods for the portable MinION sequencer (Oxford Nanopore Technologies) and the Illumina range of instruments, and a bioinformatics pipeline for generating consensus sequences. The MinION protocol does not require an Internet connection for analysis, making it suitable for field applications with limited connectivity. Our method relies on multiplex PCR for targeted enrichment of viral genomes from samples containing as few as 50 genome copies per reaction. Viral consensus sequences can be achieved in 1–2 d by starting with clinical samples and following a simple laboratory workflow. This method has been successfully used by several groups studying Zika virus evolution and is facilitating an understanding of the spread of the virus in the Americas. The protocol can be used to sequence other viral genomes using the online Primal Scheme primer designer software. It is suitable for sequencing either RNA or DNA viruses in the field during outbreaks or as an inexpensive, convenient method for use in the lab.

6 Discussion

Technology development

Benchtop sequencing instruments were introduced in 2011 and provided researchers with a cost-effective option with sufficient power to tackle small to medium sized genomes. In the study of *Pseudomonas aeruginosa* in burns, 141 isolates were sequenced on an Illumina MiSeq instrument in batches of 24 over multiple runs. A significant advantage of benchtop instruments compared with larger instruments such as the HiSeq are the shortened run time and lower running costs. This in turn makes them more amenable to being drafted into use for outbreak sequencing quickly and on demand. Despite this, total turnaround times when including the time taken to perform library preparation and bioinformatics analysis may still be as long as a week, which may be too slow for urgent investigations.

During the Salmonella outbreak the MiSeq sequencing was performed using a novel 'draft sequencing' protocol. Common run modes for the MiSeq are 150 and 250 base paired-end sequencing that take up to 2.5 days to complete. These run modes are required for achieving the maximum run yield and improve the results for *de novo* assembly applications. We proposed that for rapid SNP calling the reads only need to be long enough for most to align unambiguously so set the read length to 75 bp. Reducing the read length meant we were able to reduce the chemistry time as higher rates of phasing could be tolerated. Illumina nucleotides have a bulky side chain with a fluorescent dye which is largely responsible for phasing, a measure of the completeness

of the incorporation reaction based on the amount of fluorescence still present for the previous base. If a cluster contains around 1000 molecules a phasing value of 0.1% means 10 strands are falling behind per cycle, resulting in reducing signal to noise ratio along the read. The final modification was to image only one surface, halving the time spent imaging.

This outbreak in August 2014 coincided with early testing of the MinION sequencer. We wished to determine whether the MinION could provide clinically useful information during an ongoing bacterial outbreak. In addition to the 16 samples sequenced on the MiSeq in draft mode we also sequenced two isolates on the MinION, one from the outbreak and one unrelated strain. We first attempted this during the outbreak with the alpha version of the MinION chemistry R6 but we experienced poor results with very high error reads. We subsequently repeated the experiments using the updated R7 chemistry which yielded much higher numbers of 2D reads. The MinION analysis was therefore performed retrospectively. In order to simulate real-time data, reads were analysed as they would have become available in ten minute intervals after the start of the run. As well as having a shorter library preparation time than the MiSeq, we demonstrated two analysis methods that could rule isolates in or out of the outbreak within two hours of starting the run. This demonstrated that the MinION has an advantage in terms of speed over the MiSeq. This analysis relied on methods such as phylogenetic placement that compensated for the noisy data. Both methods were performed on DNA extracted from a pure culture which remains the rate limiting step regardless of the technology used. The sample to answer time could be in theory be significantly reduced if stool samples were tested directly using a metagenomics

approach such as during the German outbreak of Shiga-toxigenic *Escherichia coli* O104:H4[69]. It is not known however whether sequencing would generate sufficient genome coverage directly from clinical samples in order to accurately perform phylogenetic inference and this is complicated by the large number of related taxa in the normal human gut microbiome.

Prior to the *Salmonella* study, we had used single molecule PacBio sequencing to generate a high-quality reference genome for the most common cluster 'Clade E' (ST395) in the *Pseudomonas aeruginosa* burns study. Library preparation and sequencing was performed by the Norwegian Sequencing Centre in Oslo on a PacBio RSII using the P5-C3 chemistry. The sequencing cost $1000 on an instrument that retails for $750,000. $1000 is still 10x the cost of sequencing a single isolate on an Illumina platform but it generated a near-complete reference genome which we could use for very high sensitivity 'forensic-grade' variant calling. This approach permitted us to generate very high-resolution phylogenetic trees of a group of very closely related isolates to reveal microdiversity. In contrast, the simplicity of the MinION instrument means it can be provided effectively free of charge as a part of a $1000 MinION starter pack. As there are no infrastructure or instrument amortisation costs to worry about the real cost of sequencing is the same at the reagents and flowcells. In the Ebola study we sequenced one genome per flowcell without using any barcoding. This meant that the cost per sample was >$500, which is expensive but it allowed the fastest possible turnaround of as little as 24 hours. In later projects including Zika sequencing we reduced the cost by sequencing 12 samples per flowcell through the use of the native

barcoding kit. Provided the throughput is high enough samples could be further multiplexed to 96 samples per flowcell using the PCR barcoding kit.

At the 2017 London Calling conference Oxford Nanopore technologies announced that a 128 channel low-cost flowcell was in development. In this new design, the application-specific integrated circuit (ASIC) which performs low level analogue-to-digital conversion would remain on the MinION reducing the cost of the flowcell. A low-cost flowcell such as this would be ideal for viral surveillance applications where typical flowcell yields of a few gigabases are overkill for sequencing small viral genomes. Generating sufficient coverage of a single Ebola virus genome took as little as 15 minutes on a standard flowcell so would be run for 2 hours on a low-cost flowcell, while 12 Zika virus genomes which would usually take 4 hours would be run overnight. If the laborious barcoding protocol could be simplified then barcoded samples could be continuously loaded onto the flowcell when ready. The cholesterol tethers on the sequencing adapters adsorb DNA fragments to the membranes allowing new samples to be added to an ongoing run on top of the previous sample. Exploiting this with a 'run until' system guiding loading could be used in future to improve efficiency and reduce per sample costs.

Nanopore sequencing technology offers three clear advantages over existing technology; portability, low cost and long read lengths. Other technologies rely on complex optical based detection methods with lenses which need calibration after installation. In addition, all technologies require large volumes of reagents and a fluidics system to deliver them which has severely hampered miniaturisation efforts. The

MinION uses electrophysical detection so has neither of these limitations. In addition, the device draws less than one ampere so can be powered from the USB port of a laptop computer. The laptop can also provide the compute required for data collection and basecalling. This means that the samples can be sequenced close to where they were collected for the fastest time from sample to answer rather than shipping them to the sequencing facility and the difficulties associated with exporting samples.

Mobile laboratories

Genome sequencing during outbreaks can inform the public health response to infection, but only if results can be generated quickly enough. Previously, samples from an outbreak zone needed to be collected, shipped to a well-equipped laboratory, sequenced and analysed before they can be used to guide public health measures. Jeremy Farrar, director of the Wellcome Trust said surveillance "is largely stamp collecting" unless response times can be "counted in days, at most weeks, not months and years". The MinION offers the first practical real-time genome surveillance system.

For the Ebola study we assembled a mobile 'lab-in-a-suitcase' which was transported as standard airline luggage. It consists of a thermocycler, pipettes, consumables and temperature controlled reagents in polystyrene boxes. On arrival, the reagents were transferred to a fridge freezer already in the lab at Donka Hospital, Conakry. Power for the fridge freezer and thermocycler was provided by the grid with generator backup. The laboratory was able to be moved twice as the outbreak progressed to where it was most needed, both locations had mains power with generator backup. This worked

effectively during the Ebola virus outbreak because the sequencing functionality was a bolt-on to the diagnostic lab which was performed RNA extraction and RT-qPCR to provide a rapid diagnostic service. These labs were situated at Ebola treatment centres run by NGOs who built and maintained the infrastructure required. These centres take time to establish and would not be available in the initial days or weeks of a new outbreak. A truly portable surveillance system should be portable enough to access remote areas and should be able to safely process infectious material by the use of a portable isolator. Using a suitable lithium ion battery to power the equipment with a solar panel for full 'off-grid' sequencing would allow sequencing in remote areas where no power is available. We have successfully used the miniPCR, Qubit fluorimeter and minifuge on battery power in the field. Ideally all reagents should be lyophilised to allow long term storage at ambient temperature. Primers and RT-PCR reagents are readily available in lyophilised formats. These reagents will typically use trehalose as an additive which is an excellent protein stabiliser. If an amplicon sequencing method is being used then primers and reagents could be prepared and lyophilised in advance allowing for a one-tube 'resuspend in sample' type library preparation minimising the risk of contamination.

In 2015 during the Ebola outbreak the only basecaller available was the cloud service Metrichor which meant FAST5 read files needed to be uploaded for basecalling. The FAST5 files themselves were needed downstream for the event data so performing basecalling in country would not have been productive. We transferred unbasecalled files by making a TAR.GZ of 10,000 FAST5 files for each sample. The resulting ~500 Mb file was uploaded to Google Drive as this supported resuming interrupted uploads.

This file was downloaded to servers in Birmingham for basecalling using Metrichor and downstream analysis. This required the development of a new 'variants' module in Nanopolish developed by Jared Simpson and a new 6-mer pore model in order to produce accurate SNP calls. We validated the results by resequencing five samples using Illumina technology and demonstrated that the calls were 100% concordant.

Sequencing methodology

When travelling to Guinea for the Ebola study we transported reagents to perform both the amplicon method and a metagenomics approach which we didn't use beyond two trial runs as it generated poor results. Difficulties in uploading only 10,000 amplicon reads over a 3G connection meant that the metagenomics based approach was impractical in the situation. Although effective the Ebola RT-PCR scheme was still laborious, it required 12 PCR reactions to be set up, cleaned up and quantified per sample (11 regions plus a negative control). The PCR machine I transported was only a small 25 well instrument which limited the number of samples to two per day.

For Zika virus sequencing an amplicon method was chosen as we had experience with them from sequencing Ebola and we knew offered the highest sensitivity. There were reports that other groups were having difficulties sequencing Zika virus due to low viral titre in clinical samples. The modal Ct value for Zika positive patients turned out to be 36 in the large study into the establishment of Zika virus in Brazil[70] equivalent to only 10 genome copies per µl of RNA. Fragment lengths in nucleic acid extractions are usually normally distributed so having a high titre increases the likelihood of there

being long template molecules. When sequencing Ebola we used fewer longer amplicons, which was possible due to the high titre. In order to overcome the low titre problem with Zika virus we focused on short 400 bp amplicons which meant no longer exploiting the long-read capabilities of the MinION yet it was now compatible with Illumina read lengths. To generate the tiling amplicons for sequencing we would need 35 individual PCR reactions yet if we could generate the amplicons in a multiplex PCR we could combine it with barcoding to achieve 420 amplicons per flowcell or 12 Zika virus genomes. Pooling even and odd regions together allowed us to generate the amplicon products in two reactions per sample or 24 reactions per flowcell each with non-overlapping products. Making this work required designing primers with high annealing temperatures to reduce the favourability of non-specific primer interactions. We used a low primer concentration and a two-step thermocycler program with a long, combined annealing and extension step of 5 minutes. These conditions gave successful amplification up to Ct 36 which meant we had for the first time a method available which could sequence a typical clinical sample.

The benefits of amplicon sequencing are that it is robust, inexpensive and can be done in a day (and potentially much faster with optimisation). The multiplex method published describes an online tool (primal.zibraproject.org) for automated primers design and the PCR conditions for two-step RT-PCR for sequencing Zika virus and Chikungunya although the method could be applied to a broader range of viruses. Two-step RT-PCR means the reverse transcription is done in a separate reaction to the PCR. This was used for two reasons; it allows the use of random primers for the RT step but specific primers for the PCR and it is more sensitive as each step can use the optimal

buffer. We also provided an optimised library production for both MinION and Illumina sequencing. This was required particularly for MinION sequencing as the 400 bp products are considerably shorter than the typical fragment length requiring the input to be reduced to achieve the expected molarity for ligation. The barcoding process itself is still too laborious, requiring end-preparation and barcode ligation before pooling and sequencing adapter ligation. With further work It will be possible to eliminate need for a standalone barcode ligation by adding it with a second round PCR. This could be one possible path to a fully portable, lyophilised sample preparation for use in the field.

Amplicon sequencing unfortunately has many limitations chiefly that is requires *a priori* knowledge of the outbreak strain in order to design working primers. A downside of the high specificity of PCR is that it is sensitive to mismatches in the primer binding sites such would be expected in other lineages or strains. This makes it more suitable to outbreak work than to population scale surveillance efforts. In this situation bait capture may be more suitable as the baits are typically longer and therefore more tolerant to mismatches, potentially up to 30% sequence divergence. In in the case of an unknown pathogen you would have to switch to a meta-transcriptomics approach. This is usually done by filtering a sample through a 0.22 μm filter to remove cells and treating with DNAse to degrade any extracellular DNA before doing the extraction. The sensitivity of this method is dependent on the relative abundance of the virus of interest with respect to the background. Both bait capture and metagenomics methods are significantly more laborious than amplicon sequencing and may not be practical for a mobile laboratory setting. One problem highlighted by working on low titre samples was the need for meticulous contamination prevention measures which is more difficult in long multi

stage protocols than for PCR which can be set up in a hood. Dedicated PCR hoods must be used for preparing master mix and adding template to prevent amplicons contaminating subsequent reactions.

Real-time analysis

Nanopore sequencing presents a new paradigm for real-time analysis because it is the first technology that generates full length reads during the run. Most of technologies do not 'reload' so the reads are only available after the run finishes. In the Salmonella study we sought to explore how long it would take to get to the answer rather than the answer itself. To do this we generated read sets using the time stamps in the reads of reads that were available in ten minute windows. We investigated methods we expected to be robust to either noisy long-reads or low coverage. The first analysis approach was aligning reads to the MetaPhaAn database of taxon defining genes. Detecting presence or absence of genes is tolerant to a high error rate in the reads as the probability of a long read aligning by chance is very low. Using this method we could unambiguously identify the species as *Salmonella enterica* within 30 minutes. Differences between the outbreak and the non-outbreak strain were also observed after 50 minutes as three different chromosomally encoded phages were detected as we generated more coverage; Gifsy 2 in the outbreak strain and ST64B/RE_2010 in the non-outbreak strain. Two reads were identified as originating from *Citrobacter freundii* however as this is another member of the family Enterobacteriaceae it was most likely a missassigned at a lower rank than it should have been.

The second approach used phylogenetic placement to place new samples on tree build using *Salmonella enterica* and *Salmonella enterica* serovar Enteritidis genomes. With this method it was possible to determine after 40 minutes that both samples were likely from the serovar Enteritidis. The same process repeated using a reference tree containing only Enteritidis isolates, was able to assign one sample to the main hospital cluster after 100 minutes. The other sample could be assigned to a different cluster containing a mixture of phage types none of which were the same as the hospital cluster (14b), after 120 minutes. Surveillance sequencing of foodborne pathogens by WGS is an exciting development pioneered by the Food and Drug Administration (FDA) in the US[68]. Since the 1st April 2014, Public Health England (PHE) has also routinely sequenced all Salmonella isolates referred by hospitals and general practitioners to the Salmonella Reference Laboratory, Colindale. Having this data available enabled us to perform the phylogenetic placement analysis using the MinION data for the Enteritidis clade and have sufficient resolution to be able to distinguish the outbreak sample from the non-outbreak sample. For organisms other than Salmonella that do not get routinely sequenced by PHE, identification via the presence marker genes could be performed however this would lack the resolution to be able to distinguish between closely related strains. In this case only species level assignment was possible.

Surveillance sequencing

The cost and ease of sequencing bacterial genomes and the high-resolution, digital output mean whole-genome sequencing will eventually become the routine method for epidemiological surveillance. Currently most studies are snapshots and managed by

different groups of experts, usually on a pathogen by pathogen basis. Online tools such as nextstrain.org can integrate whole-genome data and metadata in real-time and provide up to date data for integration by public health investigators rather than trying to find the information in a published study. In the UK, routine surveillance sequencing is limited to food borne pathogens and *Mycobacterium tuberculosis* referred via the reference laboratory. An outbreak of *Salmonella enterica* such as the one we had, was one of the few that could have been integrated with the national surveillance data. The genomes generated by the Illumina MiSeq were compared to existing sequences in the Public Health England GastroDataWarehouse and the US GenomeTrakr service to look for national and international links. The outbreak samples were found to form a distinct cluster but this was closely related (1 SNP) to other cases form the UK. There were not any closely linked samples in GenomeTrakr which was expected as most of the food consumed in the UK is produced within the European Union. This data was a powerful tool enabling us to quickly place the outbreak in a national context. The source was eventually tracked down to a German egg producer known to authorities for frequent breaches of food safety standards. It seems likely that people will become accustomed to the power of surveillance sequencing for the species it is available for and this will lead to a shift from conventional reference laboratory identification and typing in favour of whole-genome sequencing for all pathogens.

During the Ebola outbreak data was disseminated as soon as it was available to epidemiologists working in field either via a Microreact[71] URL or later by a scripted PDF report. These contained phylogenetic trees for each cluster and incorporated metadata provided by the field lab. After an embargo period implemented on request of

the central coordination they were pushed to a publically available github repository and nextstrain.org. There were two main lineages circulating; GN1 and SL3, and multiple epidemiological transmission chains. GN1 related to early cases in Guinea and another linked to SL3 first detected in Sierra Leone in 2014. The real-time genomic surveillance data helped field epidemiologists confirm or reject the proposed transmission chains. This was particularly useful in cases where communities were difficult to access e.g. an itinerant fishing community in Boké prefecture. There were some popular rumours at the time that organs were being harvested under the guise of Ebola research (http://www.thesierraleonetelegraph.com/sierra-leone-human-tissues-and-organs-harvested-for-trafficking/) making some distrusting or even hostile to outsiders. Integration of our data with another group who had been generating genome sequencing in Sierra Leone using Ion Torrent sequencing revealed frequent cross border transmissions which was fed back to the epidemiologist on the ground. One issue that we experienced was that metadata provided from the field often required cleansing. This was partly down to the fact that information was entered into an Excel workbook which led to many different date formats and unwanted conversions to occur. The second issue was multiple spellings being provided for the same village due to variations in oral tradition. This could be overcome using a prebuilt database with fixed format fields for data entry and drop downs for any metadata which may be available from existing mapping.

Metagenomics as a way to identify potential pathogens in the hospital environment could be a powerful technique. In the burns study a thermostatic mixer value was removed from a persistently positive outlet and we were able to sample the biofilm

found inside for metagenomic sequencing. The reads obtained were aligned to the non-redundant nucleotide database using BLAST and the most abundant taxon was found to be *P. aeruginosa*. Alignment against the Clade E reference showed that 5x coverage of the genome was obtained and using a phylogenetic placement method the sample clustered with other isolates from the shower in room nine, the outlet from which the component had been removed. Without the requirement for culture positive outlets could be identified more rapidly reducing the change of a patient being exposed. Sequencing could also be used to determine if a water sample contains safe levels of *P. aeruginosa* using a spike in of known input to calibrate. Metagenomic sequencing also has the advantage of being open-ended and is not restricted as a laboratory test might be to a specific set of validated organisms. For example the individual colony forming unit assays performed individually for *P. aeruginosa*, *Legionella pneumophila* and coliform bacteria could be combined into a single test while also being able to detect rarer pathogens such as *Mycobacterium chelonae* that would not be routinely tested.

Epidemiological inference

The complexity of epidemiological interpretation is outbreak and pathogen dependent. The Ebola outbreak was likely a single spillover event from an infected bat to a child. As this represents a point source introduction followed by continued human to human transmission we can say with some certainty that all cases are descended from that index case. This provides the simplest interpretation as for each new sample the closest neighbour in the tree should represent the most recent sampled ancestor.

Zika virus originated in Uganda where it cycled between non-human primates and *Aedes* mosquitos in the sylvanic cycle, with occasional spillover into humans causing small outbreaks. The current pandemic was a result of it changing to cycling between humans and mosquitos in the urban cycle before being introduced to Southeast Asia, Oceania and the Americas. Multiple introductions and the possibility that the virus could return to the sylvanic cycle make epidemiological inference more complex. In addition the outbreak was very poorly sampled; in 2016 there were only 23 Zika virus genome sequences from Brazil limiting the resolution of phylogenetic reconstructions. We generated and additional 54 partial genomes using the amplicon sequencing method we developed and other groups generated 149 others after we published the method online[72, 73]. Combining all the datasets allowed a detailed phylogenetic reconstruction to be performed which suggested northeast Brazil being the source of Zika virus in the Americas with onward spread from there. The virus circulated undetected in Brazil was masked by the endemic dengue and Chikungunya circulating in the country. This was an important finding to meaning the baseline for microcephaly before Zika virus needed to be taken earlier. This project again demonstrated the importance of the MinION as a tool for digital surveillance. At relatively low cost, we outfitted three labs in Brazil with all the equipment required to perform Zika virus sequencing on MinION. The amplicon sequencing method is able to generate genomes from mosquitos without altering the protocol and doing so allows you to link the cases back to mosquitos trapped in a particular area. It is assumed that *Aedes aegypti* is the sole mosquito vector but this may not be the case and detection in other species could dramatically alter the areas of the country at risk. Understanding the phylogeography of Zika virus can inform public health interventions such as mosquito eradication drives

e.g. if a large city was at risk. The genome sequence can alert you to any possible mutations in the genome in diagnostic regions which could sensitivity of important diagnostic tests.

Epidemiological investigations to investigate food or water borne outbreaks can produce ambiguous results as it is more likely that diversity may be transmitted. In the *Salmonella enterica* outbreak the contaminated eggs were tracked down to a European egg producer. Although this represents a continuous point source outbreak diversity had built up in the infected flock meaning isolates recovered from patients in the outbreak may not be identical to those from the flock by whole genome sequencing if sampling is insufficient. If an isolate however falls within a 'cloud of diversity' it is still strong evidence for that being the source. Similarly, in the burns study we discovered diversity between the water outlets on the ward but not within a single outlet. This suggests the introduction of diversity via the water supply, followed by the establishment of a biofilm community in the outlets. The evolutionary rate within a biofilm should be slow as it is a nutrient limited environment and the lack of diversity within an individual outlet supports this. The total diversity we observe in the burns ward is low compared to what you might expect to find in the environment however which is suggestive of an evolutionary bottle neck as some point in the past, whether this occurred within the hospital plumbing network is not known. The diversity between outlets however provided us with a method of identifying the likely outlet from which a patient became infected i.e. a 'map of genotypes'. Phylogenetic reconstruction within the clade showed clear evidence of clustering by room and outlet. It was high enough resolution in some instances that it was possible to distinguish between the tap and shower in room 11 and

this was consistent over multiple samples. This approach of generating a geographical map of genotypes means that any subsequent epidemiological investigation could be performed very quickly. One of the things holding back routine use whole-genome sequencing in epidemiological investigations is knowledge of the populations structure of certain organisms.

Summary

We are on the cusp of an exciting revolution in genomic epidemiology systems. We have portable nanopore sequencing technology which has proven robust enough to be transported in an airline or even a space rocket hold. Fieldable methods for amplicon sequencing can be used to generate genomes from low titre samples and sequence them within in a day. Offline analysis pipelines installed on a laptop can generate consensus sequences and place new samples in the context of the outbreak allowing information to feedback into the outbreak response. There are many so advantages to whole-genome for pathogen surveillance it is inevitable that it will eventually replace conventional typing methods and continued development in the technology will enable cost effective surveillance sequencing to detect and track outbreaks in hospitals and in the field alike.

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