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Isolation of bacteriophages from water sources and testing host specificity as a novel antimicrobial agent.

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Medical Microbiology

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ABSTRACT

The emergence of antimicrobial resistance (AMR) is a growing threat to the world due to the global misuse and overuse of antibiotics. New antibiotic-resistant bacteria cannot be prevented, controlled, or killed by existing antibiotics. This has led to the search for alternative therapies, such as bacteriophages, which are ubiquitous in nature. The interest of the 'Bacteriophage' and its effects on bacteria has been present since 1915, where Frederick William Twort first discovered these 'glassy organisms'. Bacteriophages are bacterial viruses that can target and lyse bacteria by replicating within the bacterium, therefore, it could be considered as an alternative therapy to combat AMR.

In this study, bacteriophages were isolated from different rivers and tested against a panel of two-hundred bacteria: (one hundred and sixty-nine farm bovine) isolates and (thirty- one ESBL- producing) isolates from human samples. The organisms that were used in this study were *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis* and *Klebsiella* sp. Initial spot assays were used to identify phage presence in water samples. To determine the host range, spot assays were used on the isolated bacteriophages which were then tested against different isolates. This study also performed serial dilutions to determine the phage killing titre of the isolated phages and to investigate the relationship between killing titre and host range, which could be useful for phage cocktails.

Due to the COVID-19 pandemic, the analysis of this study was not fully explored, however it did show that bacteriophages were readily isolated from different river water samples using bovine and human ESBL-producing isolates as phage targets. The phages in this study were found to have a variety of host ranges and killing titre. The study also highlighted that there was no correlation between host specificities and killing titre concentrations. Potential application of bacteriophages from this study to combat AMR shows to be promising, as broad range phages with high killing titre concentrations were detected. Further phage

research is needed to validate phage therapy as an alternative route to decrease the risk of further antibiotic-resistant bacteria being introduced.

INTRODUCTION

CHALLENGING THE RISE IN RESISTANCE: Antibiotic Resistance

Before the discovery and use of antibiotics, in 1909, German biochemist Paul Ehrlich used the term 'Magic Bullet' for a chemical that would specifically target and kill pathogens without harming the host (Aminov, 2010). Although these drugs had arsenic and heavy metal compounds, they were considered to be less toxic than other chemicals. For example, one heavy metal, Mercury was successful at effectively controlling the sexually transmitted disease (Syphilis) caused by the organism *Treponema pallidium* in humans. Mercury was used in the 1940s, however, it was replaced by penicillin due to its toxicity (Aminov, 2010). Antibiotics were designed to kill or inhibit the growth of bacteria that caused infections in humans and animals (Clokie *et al*., 2011). The discovery of antibiotics had saved many lives; however, the overuse and misuse of antibiotics have increased the prevalence of antibiotic-resistant bacteria (WHO, 2021). This was predicted by Sir Alexander Fleming in 1945 (Ventola, 2015). By discovering new medicines throughout the years, toxic chemicals and heavy metals were replaced by antibiotics to treat bacterial infections. This could suggest that in the future, antibiotics might become ineffective and therefore be used in combination with alternative medications.

In 1928, Sir Alexander Fleming discovered the antibiotic effects of a fungus (*Penicillium notatum*) against gram-positive bacteria *Staphylococcus aureus* (Tan and Tatsumaru, 2015). Although his work was underrated by the scientific community, by 1940, the interest in penicillin began to increase. With the help of biochemist Ernest Boris Chain and pathologist Howard W. Florey, penicillin marked the beginning of the antibiotic era. Penicillin was used on soldiers in World War II to control bacterial infections (Aminov, 2010) and

(Ventola, 2015). However, as time passed, resistance to antibiotics started to emerge, for example, in 1962, Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in British hospitals (Lowy, 2003). Skin infections were commonly seen in patients infected with *S. aureus,* but MRSA was found to infect different organs depending on its point of entry. For example, patients infected with MRSA were susceptible to serious life-threatening problems such as bacteraemia, bacterial endocarditis, septic arthritis, and hospital-acquired pneumonia (Siddiqui and Koirala, 2020) which can be hard to control its infection and spread due to its constant evolution to existing antibiotics (Kaur and Chate, 2015). A study on antibiotic resistance patterns in MRSA conducted by Kaur and Chate stated that *S. aureus* isolates that were classified as multidrug-resistant (MDR)-MRSA, were resistant to penicillin-derived antibiotics such as amoxicillin, penicillin, and oxacillin, therefore these antibiotics were no longer effective against infections caused by MRSA (Kaur and Chate, 2015), (WHO,2019). This is one example where one organism resisted the effects of antibiotics, evolved to survive and therefore is currently causing a rise in AMR. The danger in the rise of AMR could lead to life-threatening infections which could lead to death (WHO,2019). The 'One health' approach recognises that we humans and animals share the environment and what affects one, affects the other directly or indirectly (WHO, 2021). The severity of antibiotic resistance varies in different parts of the world, depending on certain factors. These factors include overuse and misuse of antibiotics, the lack of public education on the value of completing an antibiotic course, infection control and low funded healthcare systems. Travelling with a contagious infection can also contribute to the spread of a pathogen (WHO, 2021). The World Health Organisation also stated an 'upcoming pre-antibiotic era', assuming that regardless of the severity of the bacterial infection, the world could be in danger of finding no alternative medication that can effectively treat bacterial infections (WHO, 2021). Therefore, it is very important to understand that educating the public with information on AMR can help to prevent

and regulate these factors to minimise the effects of pathogenic bacteria . It also is important that different sectors must make the effort to stop the rise in AMR to prevent severe infection and death (WHO, 2021).

Since the discovery of penicillin, many other antibiotics were later discovered and classified to help control bacterial infections (Sengupta *et al.,* 2013). The '2019 WHO AWaRe Classification Database' (Access, Watch and Reserve) is a tool that was created by the organisation to spread awareness of the one hundred and eighty classified antibiotics that are currently safe to use. Antibiotics in this database have been classified into three groups, Access, Watch and Reserve. Access group antibiotics contain forty-eight antibiotics that work on a range of common pathogens. As stated by the WHO, this group of antibiotics (Amoxicillin, Ampicillin and Tetracycline) are commonly used due to their slow resistance potential. Watch group antibiotics contain one hundred and ten antibiotics (Biapenem, Cefamandole and Oxytetracycline), which have a higher risk of resistance potential. These antibiotics should be monitored as they can cause bacterial resistance. Reserve group antibiotics contain twenty-two antibiotics that should be used as a 'last resort' due to the risk of multi-drug resistant organisms forming. Antibiotics such as Colistin, Daptomycin and Linezolid should be highly monitored (WHO, 2019). It is important to understand that the classification system of antibiotics can always change due to bacteria evolving and rapidly developing resistance to current antibiotics that were once deemed safe to be used.

The increased risk of antibiotic resistance has been present since the 1950s (Ventola, 2015). In 1945, Sir Alexander Fleming also mentioned the 'begin of an era of abuse' of antibiotics (Ventola, 2015). Many antibiotics were mass manufactured and prescribed until it was a common trait for bacteria to have resistance against these medications, despite the warnings. The AMR review mentions the use of alternative therapies to help preserve antimicrobial agents and reduce antibiotic resistance.

ALTERNATIVE NOVEL AGENTS TO COMBAT AMR: Bacteriophage revival against AMR

The AMR review by Lord Jim O'Neill discussed the potential use of alternative products to tackle the unnecessary use of antibiotics (O'Neill, 2016). The review highlighted that antibiotics alone, cannot eradicate bacterial infections and resistance. It implied that alternative products can be used to either prevent infection, reduce bacterial resistance towards antibiotics or make antibiotics highly potent against resistant organisms by blocking essential bacterial pathways. To control the emergence of AMR, current antibiotics which are successful against severe bacterial infections could be saved by developing alternative products. One alternative product mentioned in the review is the use of bacteriophage (or phage) therapy to combat bacterial infections (O'Neill, 2016).

The interest of the 'Bacteriophage' and its effects on bacteria has been present as early as 1915 (Kutter, and Sulakvelidaze, 2005) when Frederick William Twort first discovered these 'glassy organisms'. The bacteriophage is a virus that can hijack and kill a single bacterium or a range of bacteria (Davies and Davies, 2010). The French - Canadian Microbiologist, Félix D'Hérelle in 1917 (Clokie *et al*., 2011), later discovered phage potential to eradicate bacterial infections, with its ability to kill bacteria and replicate within the target cell (Keen, 2016). With the practical and independent observations made by Twort and D'Hérelle (1915-1917), global phage research studies increased in search of new information on phages and how they could be utilised to our advantage against AMR (Clokie *et al*., 2011).

PHAGE CLASSIFICATION: Adaptations, morphological features, and Lifecycles

Phages are ubiquitous in nature and highly adaptable to their surroundings, therefore, they can be easily isolated from humans, animals, soil, sediment and (marine, river, and sewage) water. (Batinovic *et al*.,2019), (Clokie *et al*., 2011), and (Kutter, and Sulakvelidaze, 2005). Phage adaptations can vary, depending on the climate it is trying to survive in by modifying structural features, adapting to bacterial defence mechanisms, or broadening host ranges (Batinovic *et al*.,2019). By investigating phages isolated from river water and host range capabilities, phages could be further analysed and genetically modified. These phages could potentially be used to prevent, control, and target bacterial infections in the future, thus helping to reduce antibiotic resistance (Clokie *et al*., 2011).

To survive in different climates and conditions that may be unfavourable, phages must undergo morphological modifications (Clokie *et al*.,2011). As phages are ubiquitous, phage morphologies are highly variable, therefore there are many different phage types. Phage classification can be achieved by electron microscopy and nucleotide gene sequencing (Comeau *et al*., 2012). According to the 'International Committee on Taxonomy of viruses'; nucleic compositions (core) found within the protein coat (capsid) of a phage head and its morphology can be used to identify these organisms (Adriaenssens and Brister, 2017). Capsomere symmetry, size, tail length, genomic material present can all vary within phage families (Figure 1). Therefore, bacteria can never be resistant to one type of phage, as phages will evolve and evade bacterial defence mechanisms quickly so that successful infection can take place (Comeau *et al*., 2012). The classification and awareness of survival techniques of the phage are important, as scientists can use this information to genetically modify specific areas of the phage genome that will improve the potential for a given phage in terms of targeting multiple bacteria, which can be used to curate a treatment (Comeau *et al*., 2012).

Other factors such as climate conditions and different bacterial populations can cause environmental stress on the phage population. Environmental stress factors can shape a phage species survival or extinction as a phage can either possess broad or narrow ranged abilities of infection (Batinovic *et al*.,2019). Phage evolution in aquatic environments is highly influenced by chemical and environmental factors such as acidity, temperature, salinity, and ionic imbalance (Batinovic *et al*.,2019). For example, in different phage species, the temperature can determine phage activity, as the temperature decreases, depending on stress factors, the rate of adsorption can decrease, therefore producing fewer phages. But, at higher temperatures in the lysogenic lifecycle, the period of latency can be prolonged, which means that more phage particles can be produced (Jończyl *et al*.,2011). The acidity of an environment can also determine the physical stability of the phage. Similarly, salinity imbalance in aquatic environments can also cause osmotic shock to the host bacteria, which could prevent phages from successfully infecting the target bacteria (Jończyl *et al*.,2011). These factors show that although one phage can thrive in one climate, another phage may struggle to survive (Jończyl *et al*.,2011). The evolution of phages occurs within the host under extreme stress, causing structural mutations. As phages are abundant in nature, different morphological structures have been discovered and classified.

With the help of electron microscopy and nucleotide gene sequencing techniques, various phage morphologies have been classified including twenty-two families of archaeal and bacterial viruses. These were then reviewed by committees such as 'International Committee on the Taxonomy of Viruses ICTV' which were added as bacterial viruses to the taxonomic database (Adriaenssens and Brister, 2017). Bacterial classification can be useful in phage host specificity. By analysing bacteria using different tools, such as Matrix-assisted laser desorption/ionisation (MALDI-TOF), similar frequency peaks can show if a strain could be genetically similar to another strain, therefore, if one bacterium is infected by a specific phage,

the other could also be infected by the same phage. It is important to be aware of the different factors, that can affect phage infections, host range specificity and phage morphology. A killing titre of phage can also be used to determine if a phage is potent in lower concentrations. A serial dilution of phage titre is performed using a constant concentration of target bacteria. Potent broad-ranged phages are desirable for phage therapies.

Bacteriophages: Their Structural Organisation and Function
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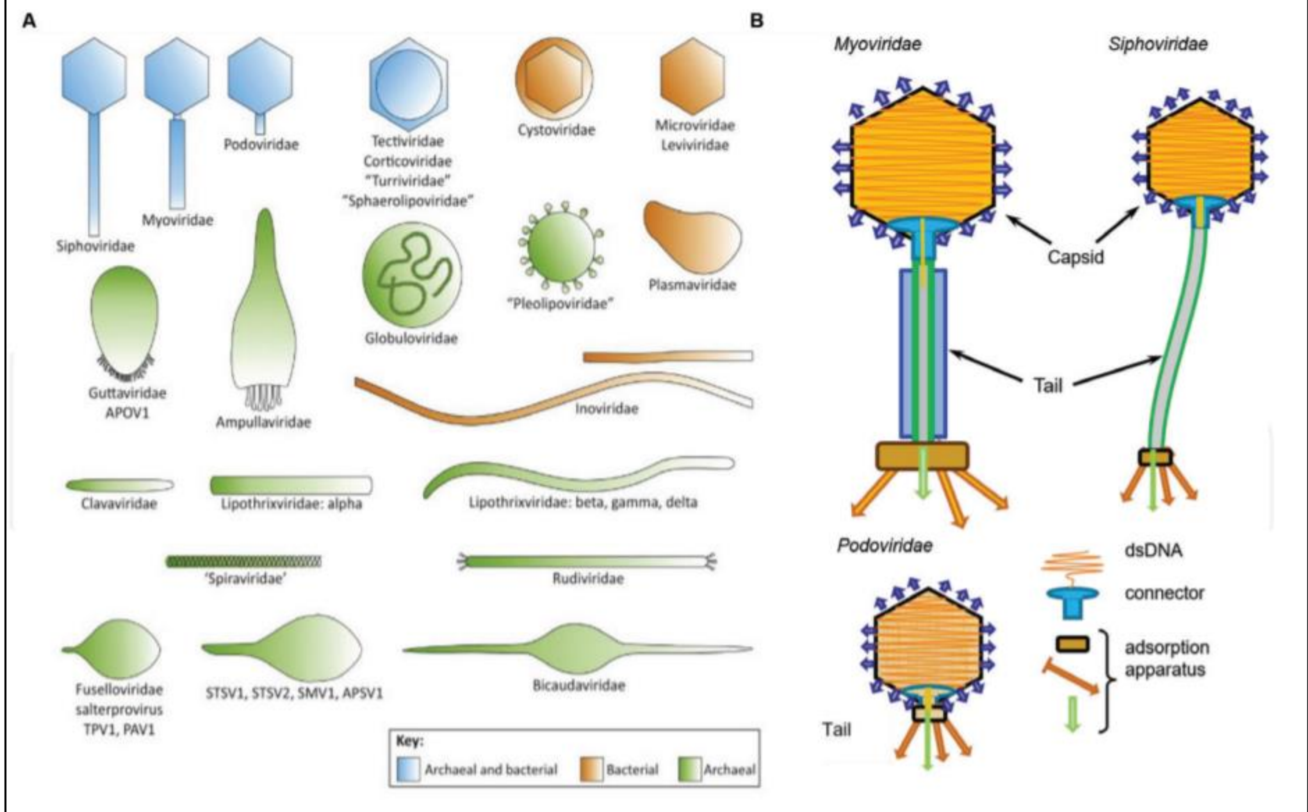


Figure 1.

A visual representation of bacteriophage classification described by (Bradley,1967). Mutations are dependent on climate conditions and environmental stress. Therefore, phages have various types of morphological structures.

BACTERIOPHAGE LIFECYCLES: How do these organisms successfully infect and replicate?

The replication of phages can be categorised into two life cycles (Lytic & Lysogenic), which are illustrated in (Figures 2 & 3). Both cycles end up with the replication and release of phages. The lytic-lysogeny lifecycle of phages is highly dependent on environmental stresses. Therefore, different phage species can go into either lifecycle to complete infection (Abedon, 2016). For example, the complex systems of bacteriophage lambda (λ) have been studied since the 1950s, where key genetic regulatory mechanisms have been unfolded. Bacteriophage lambda can follow either pathway (Oppenheim *et al.,* 2005).

For infection and reproduction to occur, the phage must first enter the bacterium host cell. Adsorption is the initial process of infection in both cycles, where depending on the species-specific phage, tail fibres can bind specifically to the receptors on the cell surface of the bacterium (Clokie *et al*., 2011). However, not all tailed phages have the ability to attach to the cell wall of the bacterium (Dimmock *et al.,* 2007). Some attach to the capsule of the cell, the flagella, or along the pili (Dimmock *et al.,* 2007). The action of binding enables the phage tail fibres to adhere and attach themselves to the bacterium. The phage proceeds to puncture a hole within the bacterial cell wall and injects the viral genomic material, ((single or double-stranded (RNA or DNA)) present in the capsid of the phage (Guttman *et al.,* 2005).

Lytic cycle (Figure 2): Once adsorption is successful; the host DNA is hijacked, and the exploitation of host machinery is in place. The process weakens the host cell as its organelles and nutrients are now used to assemble new phage particles within the bacterium. The new genomic material is packed into the capsid of the phage, as the host gets weaker. At this point, phage enzymes (lysins), are released to activate lysis on the host, killing the host directly and releasing all the new phage progeny to its surroundings, so that the process can repeat (Guttman *et al.*, 2005).

Lysogenic cycle (Figure 3): The process of circularisation occurs when the viral genomic material integrates with the bacterial DNA, creating a prophage. Phage genes present in the altered bacterial DNA (lysogens) can be transferred passively to new bacterial cells during replication. This is called 'Lysogenic conversion' (Kasman and Porter, 2018). The cycle allows phage genes to be quiescent. Therefore, the bacterial host is not harmed until the phage is exposed to environmental stress which can switch phage genes on or off and activate the lytic cycle, creating a new phage progeny. This is called 'latent infection' (Clokie e*t al.,* 2011*).* The process is not perfect and may give rise to different mutations throughout the bacterial population such as virulent traits. These traits from phage genes present in an altered DNA or specific toxin genes can contribute to antibiotic resistance (Griffiths *et al*., 1999).

As the phage interacts with a bacterium, it is dependent on certain proteins in its earlier stages to activate lifecycles for phage production to occur (Oppenheim *et al.,* 2005). Depending on the components of the phage structure, genes or lifecycle, a phage can potentially increase its potency towards both susceptible and resistant bacterial populations (Abedon *et al.,* 2011). As mentioned previously, Bacteriophage Lambda is a well-studied phage that can experience either lifecycle (Oppenheim *et al.,* 2005). These recombinant viruses can then be genetically modified to be added to medications or incorporated into disinfectants for surfaces to prevent exposure to antibiotic-resistant bacteria in future (Abedon *et al.,* 2011).

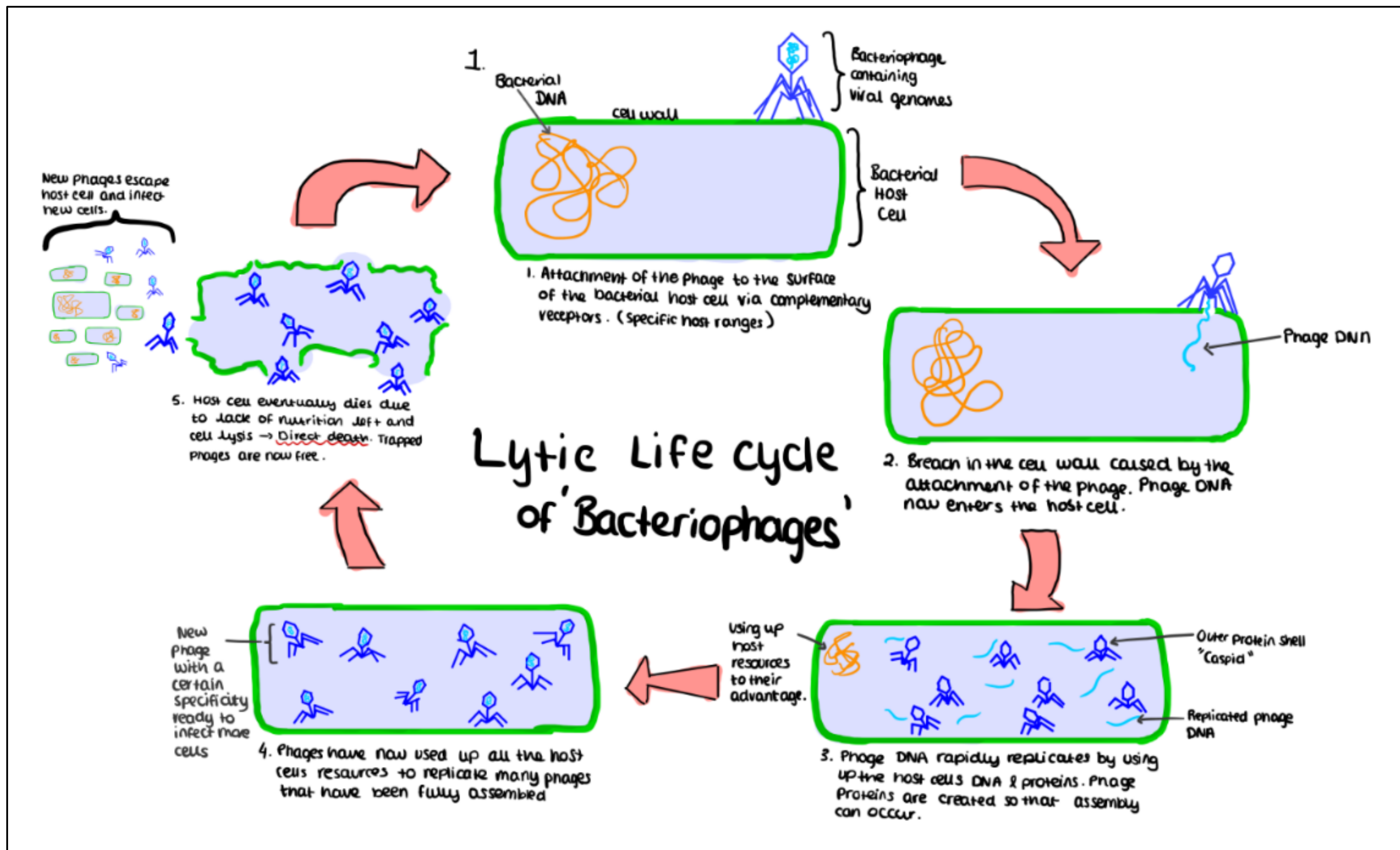


Figure 2.

A visual summary of phage infection using the Lytic cycle. The Lytic cycle of a phage directly kills the host; therefore, it is much faster and is more desirable for phage therapy. Adapted from (Clokie *et al.*,2011).

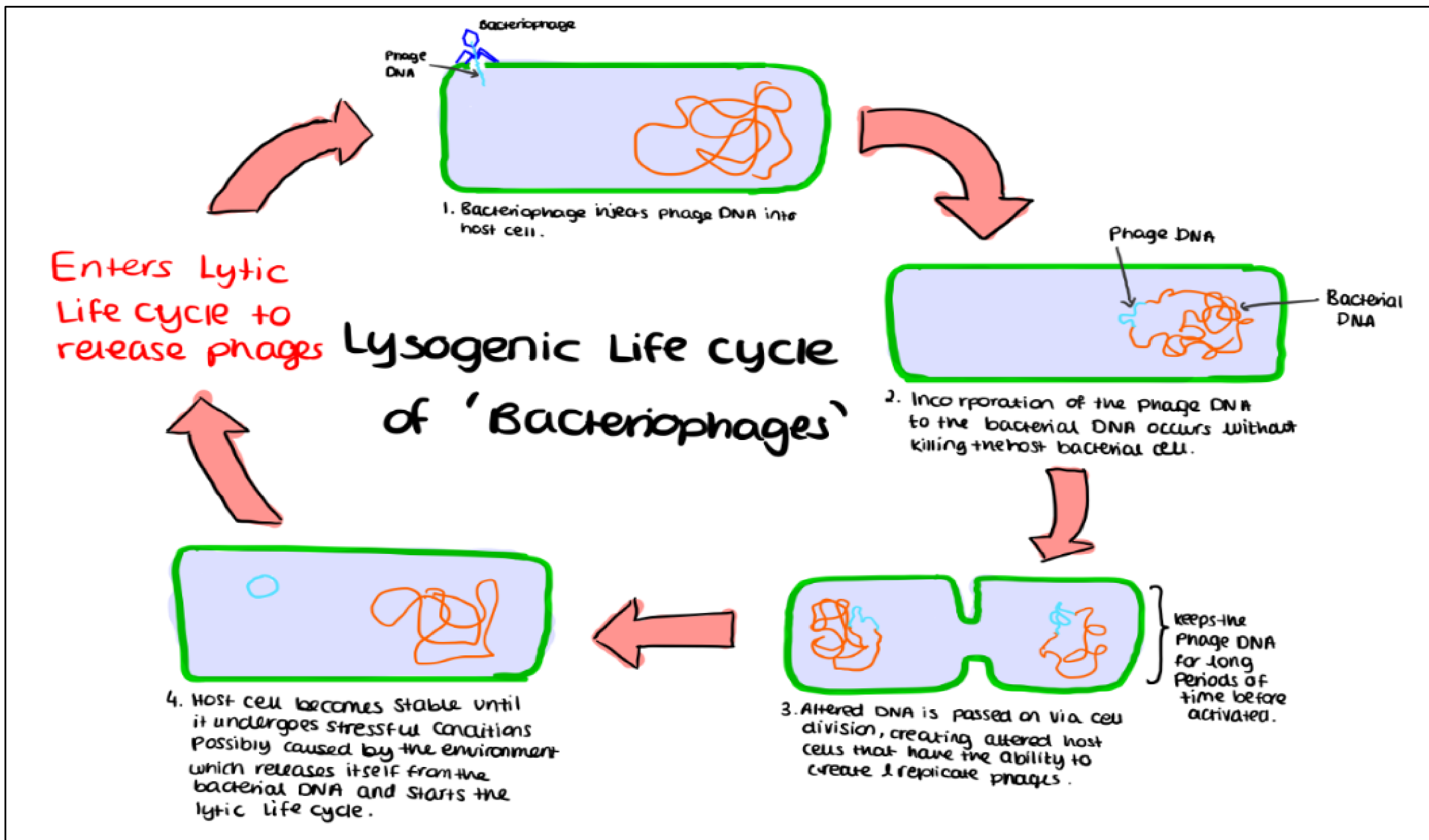


Figure 3.

A visual summary of phage infection using the Lysogenic cycle. The host is not harmed in this process. This cycle is much slower than the lytic cycle. Adapted from (Clokie *et al*.,2011).

PHAGE HOST SPECIFICITY: Against a single bacterium or bacteria

Phage host specificity is characterised by the spread of organisms a single phage can infect. Phages can rapidly mutate to survive in nature (Kutter *et al*.,2005). To survive and replicate, phages rely on the successful attachment of tail fibre proteins to receptors on bacteria. Host specificity can be classified into narrow or broad range specificity. Narrow range host specificity is when a phage binds to receptors on specific bacteria (Kutter *et al*.,2005). However, broad range phages bind to similar receptors on the cell surface of various bacterial species or strains of the same bacteria, which expands their host spectrum (Kutter *et al*.,2005). Broad-ranged phages are highly likely to survive longer than narrow ranged phages, due to their vast options, which makes them less likely to rely on a species of bacteria (Bhetwal *et al*.,2017).

Phages are dependent on the correct conditions and factors for a successful host takeover. Bacteria can build up resistance towards one phage, but it is unlikely that they can build resistance to all phages (Abedon, 2016). Phages can adapt very quickly to prevent bacteria from adjusting and adapting their defence mechanisms to resist phage infection (Kasman *et al.,*2018). Exploration of phage host range allows scientists to find out if the phage in use is acceptable to be genetically modified for phage therapy or other applications.

Host range is determined by testing the isolated phage against a variety of bacterial strains or species (Ross *et al*., 2016). Phages with hypervariability (broad-ranged phages) are more desirable for phage therapy, due to their enhanced lytic activity and infection of many different bacterial species (Kutter *et al*.,2005). For instance, in a study conducted by Ross and colleagues in 2016, the Bacteriophage Mu, infected *Enterobacter* sp*, Escherichia coli, Citrobacter freundii* and *Shigella sonnei*. In contrast, in a study conducted by Lu and colleagues in 2003, Bacteriophage JL-1, only infected *Lactobacillus plantarum* MOP3 and

Lactobacillus plantarum MOP3-M6, out of the thirty-two strains of *Lactobacillus plantarum* used in the study. These examples are one of the many phages discovered and identified with either narrow or broad host ranges, which show variety in phage species.

There are two methods of determining host specificity, by identifying 'zones of clearing' in bacterial lawns. Spotting and plaquing methods give a visual representation of either phage activity present within the sample or bactericidal effects towards phage infiltration (Ross *et al*., 2016). The turbidity of the clear zone can also be analysed (Ross *et al*., 2016). Zones of clearing with bacterial growth show that there might be different phage species present within the sample (Ross *et al*., 2016). However, it can also present as bacterial resistance to the phage species present. The visual representation of phage host specificity is very important, as it can give an insight into efficient phages that can be further researched or genetically engineered and modified to be used in future therapeutic applications (Hyman and Abedon, 2010).

Traditional methods such as Pulse-field gel electrophoresis can be used as an additional tool as described by Niu and colleagues, to further identify the DNA fingerprint of the bacteria used to isolate the phage. It can be used to compare different DNA fingerprints of bacteria to determine whether or not a phage is a narrow or broad range (Niu *et al.,*2009). However, in this project, MALDI-TOF was used to identify bacterial fingerprints of isolates used in this study, by comparing frequency (*m/z*) and mass to charge ratio (intensity, InU). MALDI-TOF is quick, cheap, and a specific tool that is currently replacing traditional methods, however when identifying unknown isolates, a database containing the spectra of known organisms is used (Rychert, 2019). The article written by Lynn and colleagues (1999), described that the Family *Enterobacteriaceae* had five specific frequency peaks 4364, 5380, 6384,6856 and 9540 *m/z*. The peaks are indicators of protein present in abundance, in the bacterial DNA fingerprint. Other known mass peaks can be compared with the data found to determine similar species or sub species of unknown bacteria (Calvano *et al.,* 2016).

CO- EXISTENCE OF PHAGE AND BACTERIA: Anti-Phage mechanisms are produced by bacteria to survive phage infections.

As bacteria and phage coexist in many different environments, it is important to understand how the microbial community is maintained and balanced. Therefore, phage defence and resistance mechanisms must be explored. The coevolution of bacteria and phage can depend on selective environmental pressures. (Oechslin, 2018). Mutations in phages or bacteria can either increase phage infectivity and specificity or create phage-resistant bacteria within the environment (Brüssow and Kutter, 2005). To balance the coexistence of bacteria and phage, each must adapt to new mechanisms to avoid total extinction (Bhetwal *et al*.,2017). Therefore, it is important to understand how bacteria survive phage infection to control replication and how genetic adaptions within the bacterial DNA can offer resistance towards phage infection.

Phage resistance can protect the host bacterial cell; however, bacteria cannot be resistant to all phages. Therefore, as a form of protection, mutations may induce minor or major changes in bacterial structure to resist foreign organisms at any stage of invasion (Brüssow and Kutter, 2005). If the bacterial host cell mutates and resists, defence mechanisms could be triggered to prevent the progression of phage infection (Lin *et al*.,2017). Different defence mechanisms can detect unusual presence within the host cell. A modified CRISPR-Cas system can provide an adaptive immunity for the host cell (Abedon, 2012). This ensures that a barrier is in place to prevent foreign genetic material to bind with the host DNA (Stern and Sorek, 2011).

Other bacterial systems such as restriction-modification systems (R-M systems) are primitive immune systems within a bacterium. The R-M system prevents foreign genetic material to combine with the host bacterial DNA. Bacteria can use methylation on foreign

genetic material which is analysed by using two enzymes: methyltransferase (MTase) and restriction endonucleases (REase) (Vasu and Nagaraja, 2013). Due to the lack of methylation within the phage genetic material, REases can identify foreign material as nonself. The nonself material is then cleaved out while MTase further identifies self and nonself genetic material within the host (Vasu and Nagaraja, 2013). This system prevents foreign genetic material to bind with the host cell, thus the process of phage infection is stopped (Stern and Sorek, 2011).

Abortive infection is the self-destruction of the host cell by lysis and can be considered as a phage resistant mechanism. Unfortunately, this process of abortive infection can occur much further into phage infection or as the phage is assembled (Oechslin, 2018). Mutations in the bacterial genome can introduce a loss or an adaptation of the complementary outer membrane proteins (cell surface receptors). As a form of protection, receptors of the host can be blocked or disguised to prevent infection by a foreign organism (Lin *et al*.,2017). This activity can block the initial attachment (adsorption) of phage tails to the bacterium (Lin *et al*.,2017). Anti-phage mechanisms of bacteria are adapted regularly to prevent the progression of any phage infection in an environment (Abedon, 2012). The coexistence of bacteria and phages in a certain environment might give bacteria the ability to develop resistance towards other phages in different environments (Stern and Sorek, 2011).

It is important to understand that using this information on bacteria defence mechanisms against phages, scientists could genetically modify the target bacteria to be less resistant towards a particular phage to increase infection. In contrast, phages could be genetically modified to maximise infection within a certain bacterial species. However, like bacteria, phages also have the ability, as their competitors, to adapt their genomes to increase the possibility of successful infection so that they can survive in different environments to prevent total extinction (Clokie *et al*.,2011).

PHAGE DEFENCE MECHANISMS: To evade bacterial adaptations.

The predator-prey rate between phages and bacteria can fluctuate depending on environmental factors. The constant complex cycle of phage resistance from bacteria and counter resistant mutations created by phages ensures that a balance is kept within the ecosystem (Høyland-Kroghsbo *et al.,* 2013). The evolution of phages provides a great opportunity for spontaneous mutations to arise (Stern and Sorek, 2011). It is important to understand the complexity of phage-host relationships and how they thrive for existence (Høyland-Kroghsbo *et al.,* 2013). Information on evolved bacterial and phage defence mechanisms can be used to genetically modify phages for phage application (Samson *et al*., 2013).

Counter mechanisms of phages have adapted to existing bacterial defence systems to successfully infect and survive within the environment (Samson *et al*., 2013). One counter mechanism produced by phages is the hydrolysation of host cell surface receptors. Once hydrolysed by degrading enzymes produced by phage genes, receptors are uncovered, giving access for phage tails to start an infection (Samson *et al*., 2013). The compatibility of fibres from tailed phages with protein receptors on the bacterial cell surface of one or more bacterium is a significant factor of successful phage infection (Oechslin, 2018). The evasion of bacterial immune pathways such as R-M systems is another form of phage counter mechanism. The modification of phage restriction sites includes the sites being spaced far apart, smaller sites present or masking specific sites to avoid detection from bacterial defence systems. With this, the phage is masked and is no longer detected by bacterial defence mechanisms, allowing for successful infection to occur (Samson *et al*., 2013). The CRISPR-Cas system can also be evaded by phages. Mutations can be introduced into the phage genome by either the substitution of a nucleotide, having anti-CRISPR genes activated or having their very own

CRISPR-Cas systems. This counter mechanism of phage can be activated to overcome bacterial defence mechanisms (Samson *et al*., 2013).

These examples of phage defence mechanisms are a fraction of what has been discovered by scientists. Phage defence mechanisms are crucial, as scientists can take advantage of this information to genetically engineer efficient phages that can be used in therapeutic applications, agriculture, food industries and many other applications in the future.

THE POTENTIAL FOR PHAGE THERAPY: Animals and agriculture

Antibiotics have helped with many aspects of animal infectious diseases and agriculture, however, with the current risk of AMR, alternative products can be considered to prevent further resistance (Rodhe *et al.,* 2018). The application of phages can potentially help in veterinary medicine, agriculture, food safety, wastewater treatments and human infections (Fernàndez *et al.,* 2018). Phages can also be used for the reduction of food spoilage, contamination, and the prevention of foodborne diseases (Sulakvelidaze and Barrow, 2005).

The use of phages dates back to 1919-1927, where Félix D'Hérelle conducted many pilot experiments on animals and humans (Clokie *et al.,* 2011). D'Hérelle first implemented the isolation and administration of phages in chickens. In France. in 1919, a large outbreak of typhoid was discovered in chickens (Sulakvelidaze and Barrow, 2005). D'Hérelle wanted to see if phage therapy could help with the reduction of this bacterial infection. At first, he isolated the bacteria (*Salmonella gallinarum*) and the phage from the dead animals (Sulakvelidaze and Barrow, 2005). To conduct a pilot experiment, it consisted of six chickens with typhoid infection. As a part of the control, two chickens were not administrated with the phage that could kill this bacterium. Successfully, four chickens that were given the phage treatment survived. He proceeded to use a larger group, which he called an 'immunisation experiment' (Sulakvelidaze and Barrow, 2005). He then infected one hundred chickens with this bacterium and administrated twenty chickens with the phage that had shown promising results in the pilot experiment. As expected, the phage treated chickens survived. This method was then implemented on other animals such as rabbits, mice, and guinea pigs with bacterial infections (Sulakvelidaze and Barrow, 2005). Many early phage studies summarised by Sulakvelidaze, and Barrow suggested that *Salmonella, Escherichia coli, Acinetobacter baumannii, Clostridium difficile, Enterococcus faecium, Pseudomonas aeruginosa, Vibrio cholerae* and

Staphylococcus aureus infections in animals could be treated with the phages that were isolated from animals that died from that infection (Sulakvelidaze and Barrow, 2005).

A report by Wall and colleagues in 2009, showed that they had collected phages from fourteen different wastewater treatments plants throughout Indiana. From each location, phages were isolated using *Salmonella enterica* serovar Typhimurium γ4234. These phages were then combined into an anti- *Salmonella* phage cocktail. In this report, a preliminary and main trial was conducted, where sixteen small pigs weighing 30-40lb were each inoculated with *Salmonella enterica* serovar Typhimurium γ4234. After the pigs were infected with *S. Typhimurium*, each pig had 5ml doses of the phage treatment or mock treatment orally administered every two hours for six hours. After three doses, faecal samples were collected to find out that five out of six small pigs had no *Salmonella* colonisation detected.

In the main trial, eight market-weight pigs weighing approximately 250lb, were inoculated with *S. entrica* ser. Typhimurium and were monitored over forty-eight hours. The pigs were then screened for *Salmonella* and 15ml of the phage treatment was administered orally, every two hours for six hours. An additional eight pigs were selected as controls, which were screened and were given 15ml of the mock treatment for the same period. All sixteen pigs were in a pen with *Salmonella* infected pigs. After three doses, samples were collected from the ileum, cecum, lymph nodes and faeces. Cecal samples of phage treated pigs showed a 95% reduction in *Salmonella* organisms (1.5 $\log_{10}$ CFU/ml) compared to mock-treated pigs (2.9 $\log_{10}$ CFU/ml). Phage treated pigs also showed a 90% reduction of *Salmonella* organisms in ileal samples (1.7 $\log_{10}$ CFU/ml) compared to mock-treated pigs (2.7 $\log_{10}$ CFU/ml). Both trials showed a significant difference in *Salmonella* counts when treated with the phage cocktail (Wall *et al.,* 2009).

Other studies of phage isolations have used 'Extended-Spectrum Beta-Lactamase' (ESBL) producing bacterial strains (Mirzaei and Nilsson, 2015). The bacteria that produce

these enzymes can be found in the bowel, however, due to their resistance to existing antibiotics, these bacteria are considered to be exceedingly difficult to destroy (Wang *et al.,* 2005). Bacteria such as *Escherichia coli* and *Klebsiella* species produce this enzyme that can cause severe infections (Wang *et al.,* 2005). In this study, Wang, and colleagues isolated phage Ø9882 from hospital sewage, which infected eleven ESBL- producing *E.coli* strains. An ESBL-*E.coli* strain that was used to determine phage Ø9882 host range, was injected into two sets of mice to induce bacteraemia. After forty minutes to 'rescue' the mice from bacteraemia, a single dose of phage Ø9882 was injected into the mice. As a control, the second set of mice was injected with just saline and LB medium without the phage. After 24hrs all phage-treated mice had survived, compared to the untreated mice that had all died. After the first dose of phage Ø9882, the next dose was delayed by sixty minutes, however, phage treated mice survived and received a lower dose. As the doses decreased, the mice would become increasingly ill (Wang *et al.,* 2005). It was very promising to see that phages could be used to kill certain ESBL strains that cause severe bacterial infections in humans.

In the 'Agro-food' sector, bactericides such as 'Agriphage' and 'Biolyse' have already yielded successful results (Fernàndez *et al.,* 2018). Certis USA is a company that specialises in biological pesticides for agricultural use such as preservation and food spoilage. They have produced bactericides 'Agriphage' containing a mixture of phages that can attack *Erwinia amylovora* on pears and apples, *Clavibacter michiganensis* subsp. *michiganensis* on tomatoes, *Xanthomonas citri* subsp. *citri* on citrus, *Xanthomonas campestris* pv *vesicatoria* on peppers and *Pseudomonas syringae* pv on tomato (Certis, 2020).

With the use of alternative products, the use of antibiotics in livestock, crops and aquaculture can be reduced even further (Svircev *et al.,* 2018). However, due to the controversy around their application in the agriculture and food sectors, many products are yet to be approved, despite their successful results (Fernàndez *et al.,* 2018). The concept of using

phage therapy in veterinary medicine and agriculture maintenance is promising. It demonstrates how versatile phages have been for many decades. Early clinical trials do show a promising aspect of these alternative products that could be implemented to reduce the risk of antibiotic resistance around the world.

THE POTENTIAL FOR PHAGE THERAPY: in humans

The AMR review by Lord Jim O'Neill outlined the potential uses of alternative products to decrease the unnecessary use of antibiotics, which contributes to the rise in antibiotic resistance. Phage therapy in humans involves genetically modified phage cultures and cocktails to specifically target pathogenic bacteria (Rhode *et al*, 2018). These phage cultures can infect and kill the bacteria that is causing severe life-threatening bacterial infections in a patient. The potential use of phage therapy was tested by many scientists before the discovery of antibiotics. However, early studies did show unsuccessful results which have contributed to phage therapy being unapproved *(*Moelling *et al., 2018).* Fortunately, these setbacks provide more time, funding, and research to go into phage therapy as a therapeutic use to prevent antimicrobial resistance. In this section, a series of early human clinical trials of phage applications will be listed and the importance of recognising these early studies as a sign to be used to combat AMR will be discussed.

One of the first scientists to have utilised phages on humans and have successful clinical studies was D'Hérelle (Sulakvelidaze, and Kutter, 2005). Once D'Hérelle accomplished treating chickens in France and various animals, he began to implement phage administrations to human patients in clinical trials (Sulakvelidaze, and Kutter, 2005). Phages were either administrated intravenously, intramuscularly, or given orally to a patient, although at this time, little was known about phage efficacy, efficiency, and limitations (Abedon *et al*., 2011). In 1919, D'Hérelle decided to start one of his first clinical trials in Paris, where paediatric patients with toxic dysentery were administrated with anti-dysentery phage preparations orally. Patients had a rapid recovery after ingesting the phage preparation (Abedon *et al*., 2011).

In Egypt (1925), D'Hérelle treated four patients that were diagnosed with the bubonic plague. After these travellers were quarantined, 0.5ml of phage culture preparation named 'Pestis Bacteriophage' was injected into the patients. Patients recovered rapidly and after the

dramatic success of this clinical trial, D'Hérelle managed to apply many clinical studies around Europe and India for patients infected with cholera. In 1927 'The cholera study' in Kolkata, India, was conducted in hospitals to reduce the incidence of cholera. Anti-cholera phage preparations were first administrated to twenty-seven patients, which had given encouraging results as fatalities were reduced from 30% to 0% (Sulakvelidaze, and Kutter, 2005). As conventional treatments and less effective medications for bacterial infections decreased, the search for phage therapy increased. Sulakvelidaze and Kutter described the early studies which had been conducted in different parts of the world, for example, in the 1940s, a scientist named Walter Wart, injected Vi-phage cultures into mice with *Salmonella* Typhi. Due to its successful results of 6% mortality rates in the phage treated mice, the phages used in this previous experiment were then used in human typhoid infection. One study by Knouf and colleagues in 1946 used phage therapy on patients with typhoid infection and described that phage treated patients had a reduced mortality rate from 20% to 5%. The condition of the patients that were treated by phages rapidly improved (Sulakvelidaze and Kutter, 2005).

Phages can also infect ESBL-producing bacteria as mentioned previously. ESBL-producing bacteria are resistant to a range of β-lactam antibiotics, therefore infections caused by these bacteria are very hard to treat. An example of ESBL-producing bacteria is *Shigella*, which causes acute gastrointestinal infections such as shigellosis. A study conducted by Shanin, and colleagues, used a phage cocktail created by bacteriophage vB_SflS-ISF001 (Phage 1) and vB_SsoS-ISF002 (Phage 2) against multidrug-resistant, ESBL-producing *Shigella* species (Shanin *et al.*, 2019). Although the study used a collection of seventy isolates, only twenty-six were ESBL-producing *Shigella sonnei* and twelve were ESBL-producing *Shigella flexneri.* Both (Phage 1) and (Phage 2) and were able to infect sixty-six isolates of non-ESBL and ESBL- producing *Shigella* species when combined into a cocktail. Phage 1 was able to infect eleven ESBL-producing *Shigella sonnei* and Phage 2 was able to infect twenty

ESBL-producing *Shigella flexneri*. It is important to understand that these results show that both phages were broad range and highly potent, which is very interesting if they were to be used in phage therapy to treat multidrug-resistant *Shigella* infections in the future (Shanin *et al.*, 2019).

In earlier clinical trials, described in Slopek's series of reviews summarised by Sulakvelidaze, it provided a list of different administrative ways phages were delivered to a patient (Sulakvelidaze *et al*., 2001). Phage cultures were administrated into the nasal passage, intravenously, middle ear, to eyes via eye drops, orally, and as dressings directly applied to wounds of a patient (Sulakvelidaze *et al*., 2001). Past clinical trials and research studies suggested that phage administrative studies were wildly popular and cured many bacterial infections (Sulakvelidaze and Kutter, 2005). Unfortunately, after the discovery of antibiotics, phage studies decreased around the western world (Sulakvelidaze *et al*., 2001).

Early clinical trials of phage therapy in humans encouraged ongoing research to use phages as an alternative product to manage life-threatening bacterial infections (Patey et al., 2018). The possible use of compassionate phage therapy could be used as a last resort treatment, to patients not being able to recover from their conditions with existing medications (McCallin et al., 2019). It is important to understand that a patient's life should be saved with all available resources. The approval of compassionate phage therapy was and still is extremely controversial (McCallin et al., 2019). Possibly in the future, phage therapy could be combined with other antimicrobial treatments to treat severe bacterial infections without causing a rise in AMR (Patey et al., 2018) which could be used in desperate measures as it is currently not fully approved by health organisations such as the Food and Drugs Association (FDA) (Moelling et al., 2018).

Although phage therapy proved to eradicate bacterial infections in animals and humans, its interest declined in the western world due to the lack of knowledge of phage dosage, storage,

viability, and other additional information that affected treatments and protocols *(*McCallin *et al.,* 2019). Unknown information about infectivity, phage structure, narrow specific host range and the lack of faith in this alternative therapy all contributed to its dismissal (Clokie *et al*., 2011). Although interest was absent by hospital staff and other scientists, the interest in using phage therapy as an alternative product has arisen again as a result of AMR (O'Neill, 2011). It is important to understand that phage therapy is considered to be an upcoming alternative therapy that could be used to target bacterial infections, however, it is yet to be approved by regulatory agencies. For this reason, the use of phage therapy to the public is further delayed as time goes by. However, case by case, the FDA has approved a few clinical trials in the United States (LaFee and Buschman, 2019).

ADVANTAGES AND DISADVANTAGES OF PHAGE THERAPY

The increasing interest in phage genetics and therapy has led to the comparison of this approach with antibiotics to eradicate bacterial infections in humans, without the risk of resistance. Many scientific authors identify and evaluate the benefits and drawbacks of phage therapy (Carrillo and Abedon, 2011). For example, the reduced disruption to the natural flora of humans is one of the key advantages of phage therapy (Nilsson, 2014). This aspect of the therapy also provides a lower toxicity rate towards healthy bacteria present (Nilsson, 2014). In contrast, one disadvantage of using antibiotics is that these medications are non-selective, therefore they can target the specific bacteria intended to kill but also target the beneficial bacteria within the patient. With this quality, the patients' microbial balance can be disrupted and cause side effects as well as secondary infections (Sulakvelidaze and Kutter, 2005).

Another advantage of phage therapy is that it can be easily isolated from any environment (Carrillo and Abedon, 2011). Therefore, the production of phage therapy medications can be relatively time-saving and inexpensive, if the phages are broad-ranged and if they can be easily manipulated (Carrillo and Abedon, 2011). On the other hand, phage therapy could be time-consuming and expensive if the phage that is isolated is narrow ranged, therefore it may have to be genetically modified to have desirable characteristics and tested for safety (Carrillo and Abedon, 2011). Although the production of new antibiotics can take a long time to accomplish, they are easily approved by regulatory associations unlike phage therapy, which is yet to be approved (Carrillo and Abedon, 2011).

Phage molecules can be utilised as a vector or delivery system allowing genetically modified phage genes to alter bacterial DNA (Sulakvelidaze and Kutter, 2005). This can be done to increase the sensitivity of the bacteria towards an antibiotic or to promote host cell death as a preventative measure of resistance by delivering endolysins which could be achieved

in the lysogenic lifecycle (Sulakvelidaze and Kutter, 2005). It can also be considered to be used alongside antibiotics treatments. Another advantage of phage therapy is that its applications can vary, depending on its purpose. Phages may be used in the form of disinfectant sprays as phage cocktails on surfaces or in agriculture management such as the 'Agriphage' mentioned previously (Abedon et al, 2011). Treatments can be used either at home or hospital surfaces to prevent the exposure of antibiotic-resistant bacteria (Carrillo and Abedon, 2011). Phage treatments could be applied to the skin as a topical medication for skin infections, injected intravenously or intramuscularly for a faster route to the bloodstream or taken orally (Morozova et al., 2018).

The application of phages as a biocontrol agent applied to food safety and water treatments could also help with the limitation of exposed resistant bacteria and hospital-acquired infections (Smith, 2014). Phage cocktails are a combination of phage strains, that are present in a single dosage. In this form, phage cocktails can effectively target and kill multiple bacterial strains or species within a population. These are very efficient; however, they must be carefully engineered to prevent the passing of phage resistant genes to bacteria during infection. This could cause bacterial resistance towards the phage being used, making the treatment less effective (Carrillo and Abedon, 2011). Phages are highly diverse which may be difficult to find the correct broad lytic phage combinations in an environment (Clokie et al., 2011). For the treatment to effectively work, a wide set of host range phages must have the ability to kill multiple bacterial strains or species (Carrillo and Abedon, 2011). Although phages are abundant, not all phages that are isolated can be used in the production of cocktails due to the hypervariability in species and host range (Carrillo and Abedon, 2011). Whilst there is a promising outcome of phages being utilised to combat bacterial infections, there are still uncertain aspects of the phage. Even though phages do not disturb the normal flora of the

human, they can indirectly contribute to increasing the risk of existing bacterial infections within the body (Hyman and Abedon, 2012).

Phages that undergo the lysogenic life cycle usually contribute to the fuelling of pathogen evolution, as the phage genomic material is incorporated with the bacterial DNA, creating a bacterial lysogen (Hyman and Abedon, 2012). Generalised transduction is the packaging and transfer of any random fragment of the host bacterial genome. This type of transduction was first discovered in *Salmonella* phage P22. Phage terminase enzymes recognise a (pac) phage-specific packaging site on the DNA and cleave the genomic material to be inserted into phage heads (Chiang *et al.*, 2019). Specialised transduction is the packaging and transfer of specific fragments of the host bacterial genomes. This type of transduction was found in coliphage λ. Coliphage λ commonly uses (cos) terminase enzymes (Chiang *et al.*, 2019). Depending on the phage isolated, it can undergo either transduction, however, these processes are commonly seen as mistakes caused by the phage (Chiang *et al.*, 2019).

It is important to understand that bacteria can exploit phage transduction methods to evolve and adapt to resist attacks from both phages and antibiotics currently present. Although highly unlikely, it is important to note that virulent factors within the phage could be incorporated into new bacterial DNA (Salmon *et al.*, 2019). This could increase the risk of new resistant strains in nature. Unfortunately, this could mean that existing antibiotics might fail to work on infections caused by these new strains.

In contrast, this information can inform scientists working on phage therapy treatments that phages become 'phage criminals' if they are not mediated or engineered properly (Hyman *et al.*, 2012). However, the advantages of phage use do outweigh the risks of using these organisms as a form of prevention and treatment in human bacterial diseases (Carrillo and Abedon, 2011). It is important to understand that depending on the phage ecology, efficacy and host specificity, phage production can be time-consuming and expensive, which may be

one reason why regulatory associations have not approved this therapy. As mentioned above, phage therapy has been highly controversial due to the lack of safety information, however, an interest in this therapy has arisen again as a result of AMR (Rohde et al.,2018).

AIMS

The role of bacteriophages as a factor to decrease antimicrobial resistance has been a controversial topic. However, with the rise in AMR caused by antibiotics, scientists are researching bacteriophage therapeutic uses to stop this. This study aimed to identify the presence of bacteriophage particles within multiple water samples. Phages will be used against a range of bacteria including *Escherichia coli*, *Proteus vulgaris, Proteus Mirabilis, Pseudomonas aeruginosa* and *Klebsiella* sp (isolates from bovine faeces) and Extended-spectrum Beta-Lactamase (ESBL) producing *E.coli* strains (isolates from human samples). The isolation of phages from water samples as well as its host range specificity was investigated. Serial dilutions of certain phages were also performed to find killing titre concentrations and MALDI-TOF was used to identify possible host specificity.

OBJECTIVES

- Isolate bacteriophages from water samples and test against a range of bovine faecal and human isolates
- Test host range specificity of phages found from initial infection using spot assay and MALDI-TOF.
- Determine killing titre of phages and range of concentrations found using serial dilutions.

MATERIALS AND METHODS

Bacteria isolates from bovine faeces

The main organisms used in this study were *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis* and *Klebsiella* sp. Bacteria were isolated from bovine faeces collected from different farms: Dan Williams, Binningtons (BIN & F2), and Lynwoods. isolates were provided by Kingston University laboratories. Table 1-3 in Appendix B show (in total two- hundred isolates) one hundred and sixty-nine isolates (isolated from bovine faeces) and Table 4 show thirty-one ESBL- producing isolates (isolated from human samples).

Water Sample Collection

Water samples were collected from different rivers at seventeen locations shown in Figures 4,5,6 and 7 using clean, food grade, biodegradable water bottles that were bought and emptied just before collection. Samples were collected from Hogsmill River, River Thames, River Mole and River Wey. Water samples from four locations were collected near wastewater treatment plants (Red circles). Water samples from six locations were collected near open green fields (Light blue circles) which could be close to nearby farms and samples from seven locations were collected closer to the local area (Orange circles).

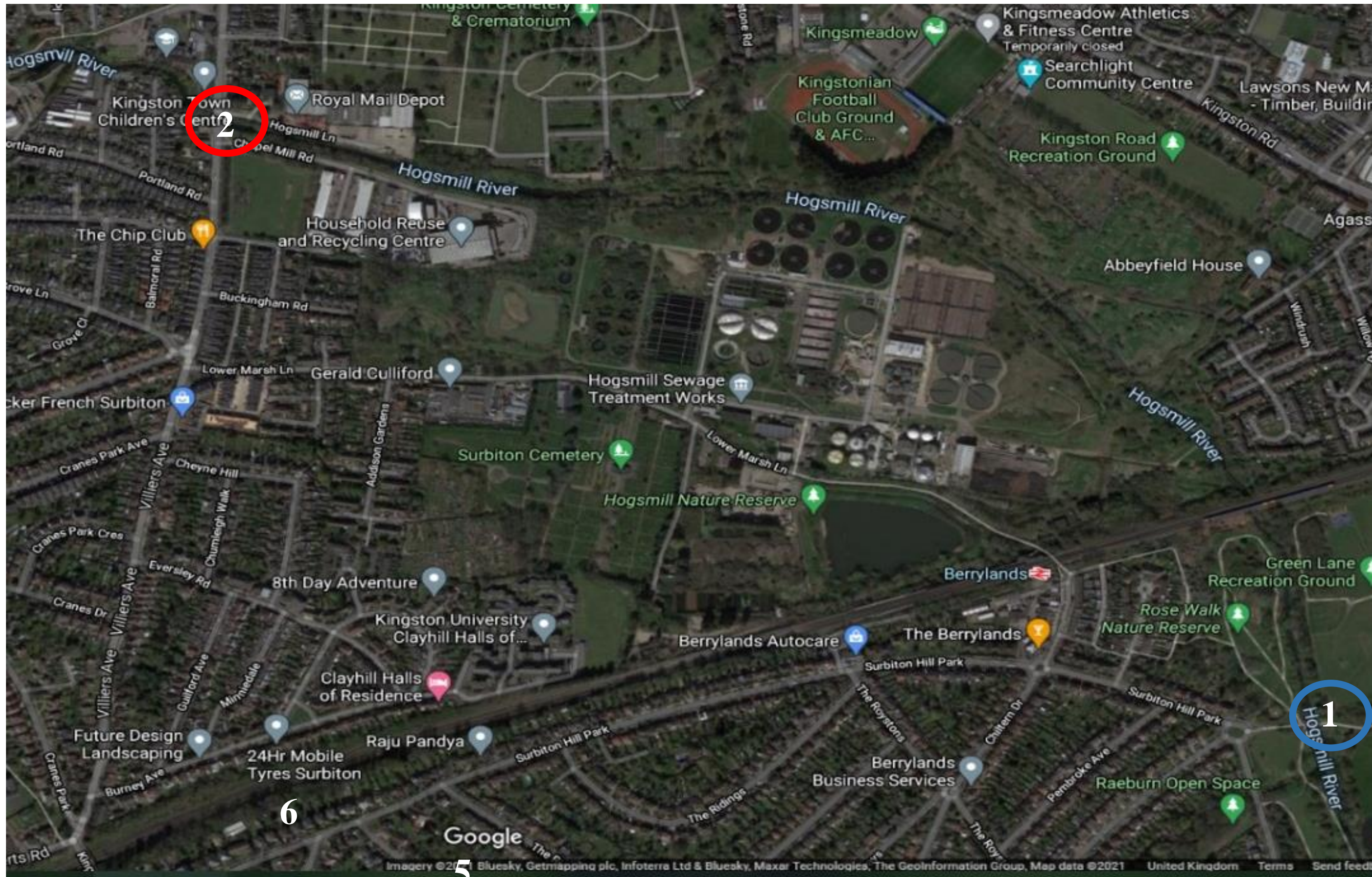


Figure 4.

Map of Hogsmill River where water samples collected using clean water bottles; **Location 1**: Upstream (U), **Location 2**: Post office (P).

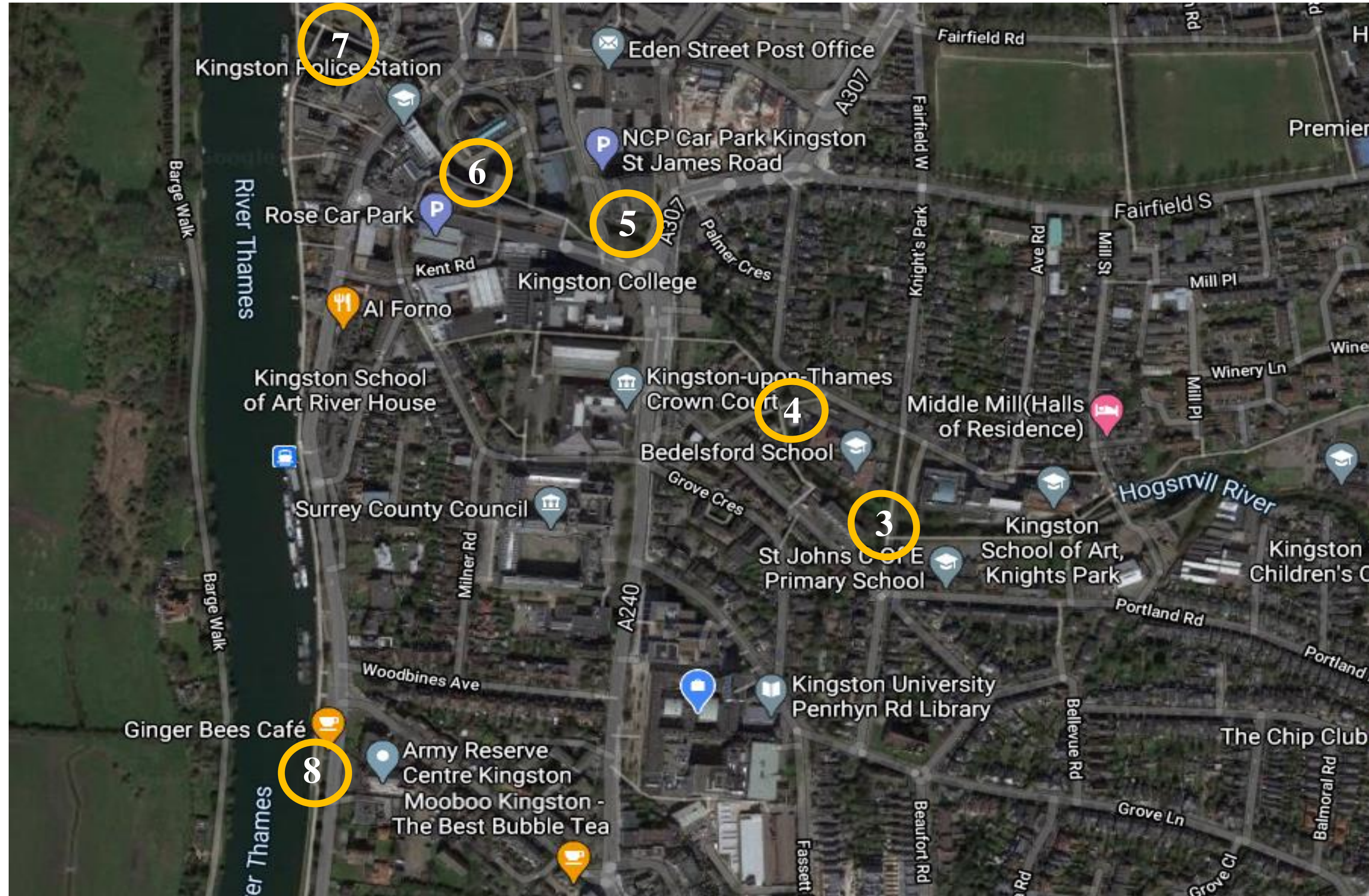


Figure 5.

Map of Hogsmill river where water samples were collected using clean water bottles; **Location 3**: Blue bridge (B), **Location 4**: Valley Walk (VW), **Location 5**: Kingston college (K), **Location 6**: Rose Theatre (R), **Location 7**: Downstream (D) and **Location 8** :River Thames sample (T).

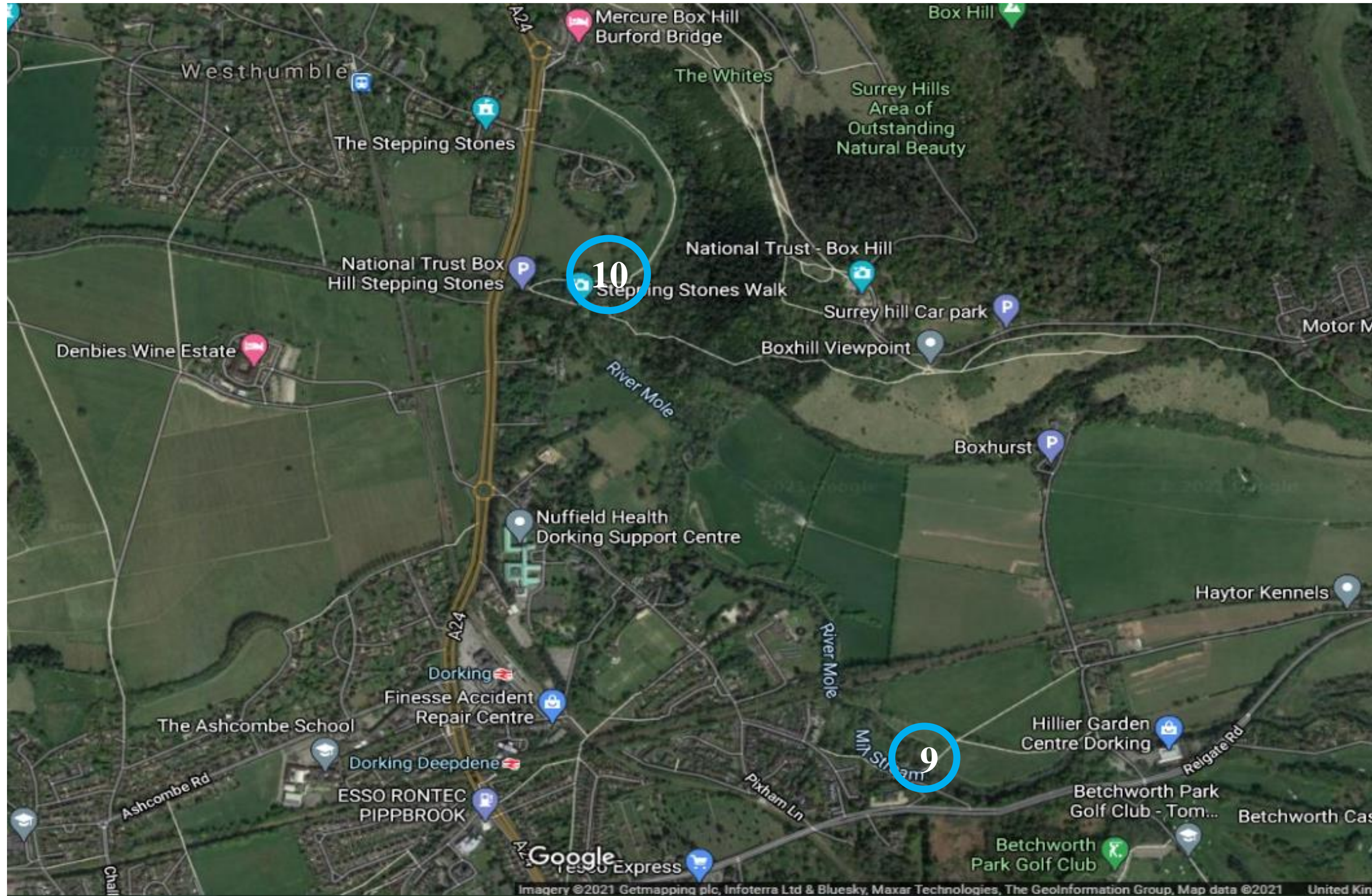


Figure 6.

Map of River Mole where water samples were collected using clean water bottles; **Location 9**: Upstream (U), **Location 10**:Stepping stones (S).

River Mole

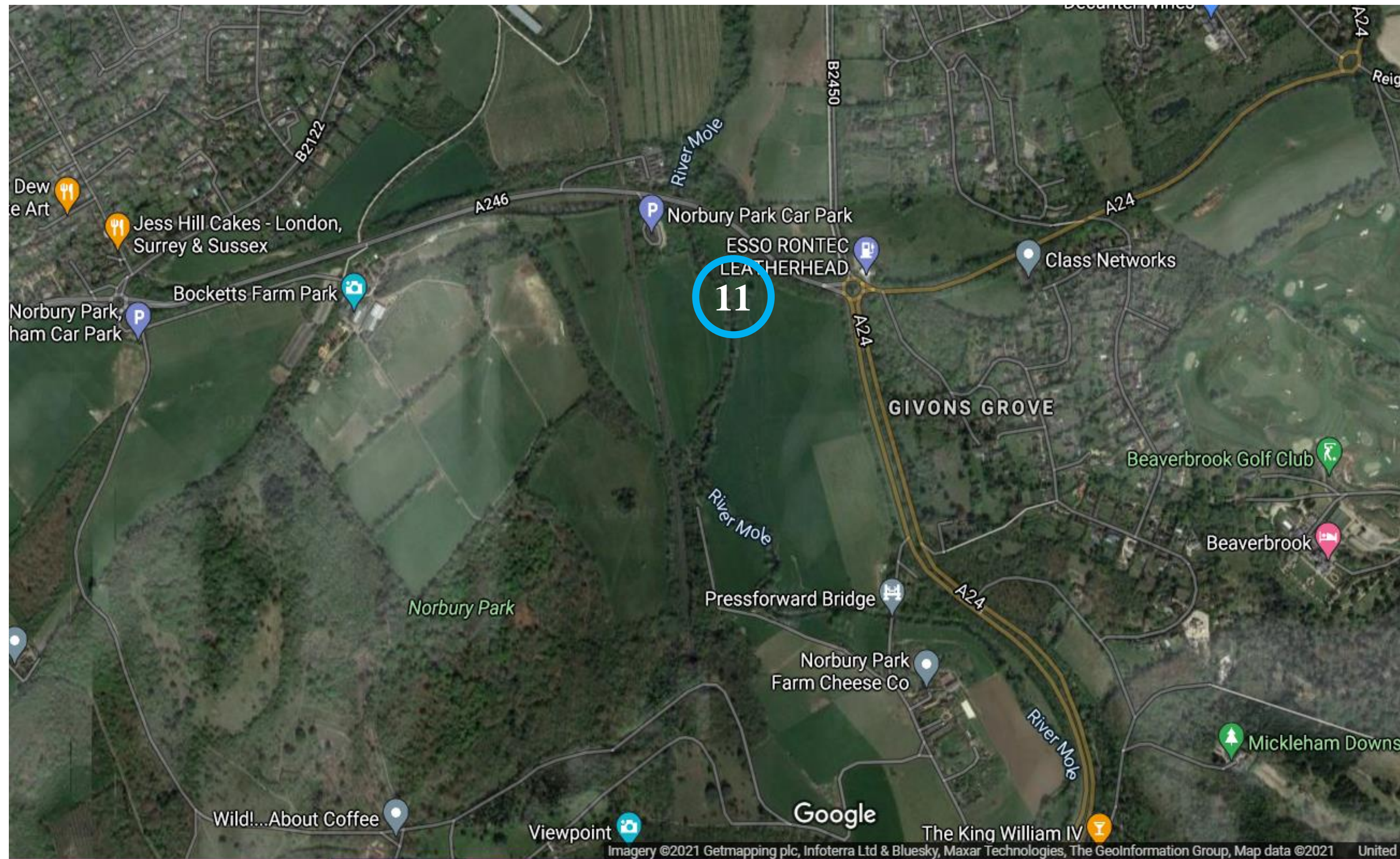


Figure 7.

Map of River Mole where water samples were collected using clean water bottles; **Location 11** : Downstream (D).

River Wey

Figure 8.
Map of River Wey where water samples were collected using clean water bottles; **Location 12:** Direct sewage outlet (S), **Location 13**: Wisley lane (B), **Location 14**: Canal up the pub (UC), **Location 15:** Canal down the pub (DC).

River Wey

Figure 9.
Map of River Wey where water samples were collected using clean water bottles; **Location 16:** Golf course and **Location 17:** Abbey Stream

Bacteriophage Detection: Preparation of culture

Aliquots of 20mL of nutrient broth were added to each sterile falcon tube. Water samples of 5ml were aseptically added to each tube. Samples were individually inoculated with one of five bacterial strains (*E. coli*, *P. aeruginosa* and *P. vulgaris, P. mirabilis* and *Klebsiella* sp) for initial samples. The same method of preparation and inoculation was applied to other clinical isolates to test for host range infectivity (Table 1-3). All the samples were incubated at 37°C for 24hrs to allow bacteria to grow aerobically.

Preparation of bacteriophage supernatant

Inoculated samples in falcon tubes were centrifuged for 10 minutes at (1448 x g). After centrifugation, samples were refrigerated for five minutes until all other samples were ready to be filtered. Samples were filtered with a Millipore 0.2µm cartridge and collected into sterile 7ml Bijoux bottles, then refrigerated until it was ready to be used.

Isolation and preservation of bacteriophages

Isolates were prepared before the sample day by streaking on nutrient agar and incubated overnight at 37°C. The bacterium was suspended in a ringer solution equal to 0.5 McFarland Standard (1.5 x 10^8 CFU /mL), then sterile cotton swabs were used to transfer bacteria to a nutrient agar plate, swabbing in three different directions, covering the whole plate. To find out the host specificity of phage present in the sample, a total of 5 x 10µl aliquots of viral supernatant, were dropped onto the nutrient agar plate containing the same bacteria used for inoculation and the plates were left for three minutes to dry. The nutrient agar plates were then incubated at 37°C aerobically overnight.

The identification of phage activity was noted by observing zones of clearing within the bacterial lawn. The zones were aseptically removed using a sterilised loop or tweezers to create an agar block which was placed into bijoux bottles with 3ml of nutrient broth. Glycerol (2ml)

was added to the bijoux bottles before being frozen at -80°C. The same bacteria used for phage isolation was added to a 5ml ringer's solution and suspended to 0.5 McFarland Standard. The bacterium was then transferred to a falcon tube containing 20ml of nutrient broth. Phage samples were completely defrosted at room temperature to extract the agar block. The sample was vortexed for ten seconds to disturb the contents inside. The agar block was aseptically removed or 500µl of the solution with the dissolved agar block was taken out. The block or solution containing phages were aseptically added to falcon tubes previously prepared. The inoculated sample containing the phage was then incubated overnight aerobically at 37ºC.

Identifying bacteriophage host specificity on different bacterial strains

To identify whether the phages found from the initial screening were broad or narrow ranged, phages were tested against multiple bacterial strains (Table 1-4). After phages were defrosted, phage blocks or supernatant were added to a falcon tube already containing 20ml of nutrient broth. Instead of inoculating a phage with one corresponding bacterial strain, twenty bacterial strains were added to the sample (Bacterial cocktail). The falcon tube was then incubated at 37ºC overnight. The next day, the sample was centrifuged as described above and filtered. To determine the phage host range, a new plate was used for each bacterial strain used in the cocktail. Each plate was swabbed with one bacterial strain used in three different directions. The supernatant derived from the sample was then dotted (total of 5 x 10µl aliquots of viral supernatant) onto each plate and left overnight to incubate at 37ºC.

Killing Titre of isolated bacteriophages

To a falcon tube, 5ml of nutrient broth and a phage block was aseptically added. Bacteria used to isolate phage was suspended in 5ml Ringer's solution to 0.5 McFarland Standard (1.5 x 10^8 CFU /ml). The inoculated Ringer's solution (1ml) was aseptically added to a falcon tube which was incubated overnight at 37ºC. Once centrifuged for 10 minutes at (1448 x g) and filtered, the supernatant was labelled as 100% neat phage. Each phage was serially diluted in nutrient broth, repeated three times, and averaged. In a sterile 96-well microtiter plate, rows A and B have positive and negative controls. The positive control is the bacteria and nutrient broth, and the negative control is the phage supernatant and nutrient broth. Rows C, D & E are labelled as phage 1 and rows F, G & H is be labelled as phage 2 . Each column was labelled as the dilution concentrations of 10^{-1} to 10^{-8} pfu/ml. Column 1 is filled with 200 µl of neat phage 1 or 2. Columns 2 to 12 is filled with 160µl of nutrient broth. Using an automatic pipette and sterile tips, 16 µl of 100% neat phage from column 1 was transferred to column 2 and mixed. This step was repeated throughout all columns (transferring 16 µl from column 2 to column 3 etc.) until 10^{-8} concentration was reached. Ringer's solution was again inoculated with bacteria and vortexed to 0.5 McFarland Standard (1.5 x 10^8 CFU/ml). The inoculated ringer's solution (10 µl) was aseptically added to each well and mixed.

The same Ringer's solution that was inoculated with bacteria, was spread onto a nutrient agar plate and left to dry for 3 minutes. The plate was labelled with the corresponding phage dilution and bacteria. An aliquot of 10 µl of diluted supernatant was aseptically dotted onto each plate. Both agar plate and microtiter plate were incubated overnight at 37ºC. Zones of the clearing were stored as mentioned above and concentration of phage efficiency were recorded.

MALDI-TOF analyses

To analyse bacterial DNA, thirty- four clinical isolates from Table 1-4 were used and sent to Great Ormond Street Hospital and analysed by Dr Francis Yongblah.

RESULTS

Total Phages collected

A total of one hundred and eleven phages were isolated from four different river samples (Table 6 & 7) in Appendix B. Different host specificity of phages were discovered using bovine faecal isolates and human (ESBL) producing *E. coli* isolates (Table 1- 4 found in Appendix B). In this study, Hogsmill river was tested seven times, River Mole was tested three times and River Wey was tested two times. The results show that as Hogsmill River was visited the most, more phages were found at this site compared to the other sites. In this current study, Table 6 shows that sixty-one phages were isolated at different locations of Hogsmill river using bovine faecal isolates (Table 1). Of the sixty-one phages, twenty-nine of the phages had infected seventeen known *E. coli* strains (Table 1). Only one phage infected one known *P. aeruginosa* strain found in (Table 3). Sixteen phages infected eight known *P. vulgaris* strains, two phages also infected two known *P. mirabilis* strains (Table 3). Finally, two phages infected one known *Klebsiella* sp (Table 3). Of the Hogsmill river samples, *E. coli* strains; Bin 312B, F2 16B, F2 8C and *P. vulgaris* LV3 11B were more susceptible to phage infection.

Of the River Mole samples in Table 6, twenty-four phages were isolated using the clinical isolates from (Table 1). Fifteen phages infected eight different strains of *E.coli* and three phages also infected one *P. aeruginosa* strain (Table 3). Six phages had infected two different strains of *P. vulgaris,* but no phages had infected any *Klebsiella* strains. Only one sample was taken from River Thames, and three phages had infected three different strains of *P. vulgaris* (Table 2).

At River Wey, six phages had infected one *E.coli* strain from (Table 1) and eleven phages were found to infect several *E. coli* samples. Table 7 shows *Pseudomonas* species isolated from two water samples were found to be infected by four phages. Lastly, two

phages were found to have infected one *P. vulgaris* strain found in Table 2. Of the phages found at River Wey in Table 6, *E. coli* 9F was found to be more susceptible than other strains used.

Host ranges of phages isolated using bovine isolates.

Host specificity can determine whether a phage is narrow or broad-ranged. To determine the host specificity of phages isolated from water samples, phages were further tested against different bovine faecal isolates. Table 8 and Figure 10 represent the phages that infected one or more bacteria found in Table 1 and 2.

Firstly, of the sixty-one phages isolated from the Hogsmill river, fourteen phages (Shown in red in Figure 10) were able to infect more than one bacterium. Figure 10 also suggests that phages HV4 P56 (B), HV2 P74 (K), HV2 P76 (K) MV3 P68 (S), MV2 P69 (S), MV2 P71 (D), WV1 P103 (S), WV2 P104 (UC) and WV2 P105 (UC) infected one bacterium besides its original target bacteria. However, phages HV5 P77 (VW), HV6 P87 (VW) and HV1 P1 (B) had infected only two other bacterial strains. This suggests that these phages might be narrow ranged phages as they have only infected less than two bacterial strains.

Phage HV2 P3 (B) was shown to have infected eight different *E. coli* strains and one *P. vulgaris* strain. Phage HV2 P25 (VW) was shown to have infected seven different *E. coli* strains and one *P. vulgaris* strain. Phage HV1 P6 (VW) infected six different *E. coli* strains and one *P. vulgaris* strain. Phage HV2 P26 (VW) had been shown to have infected five different *E. coli* strains and one *P. vulgaris* strain. Phages HV2 P2 (B), HV2 P5 (B) and HV1 P27 (U) had infected five different bacterial strains. All three phages had infected one or more *P. vulgaris* strains. Phage HV5 P80 (VW) and HV2 P4 (B) had infected only three bacterial strains, one of

which is a *P. vulgaris* strain. This suggests that these phages might be broad range phages as they have infected five or more bacterial strains.

Of the isolates used, only thirty-five bacteria were susceptible. Out of the thirty-five bacteria, *E. coli* BV3 23E had ten phages it was infected by, *E. coli* Bin23E had eight phages infected by, *E. coli* DWV1 25D had seven phages, *E. coli* LV3 15C and *E. coli* LV3 21B both were infected by six phages (Table 8 in Appendix B).

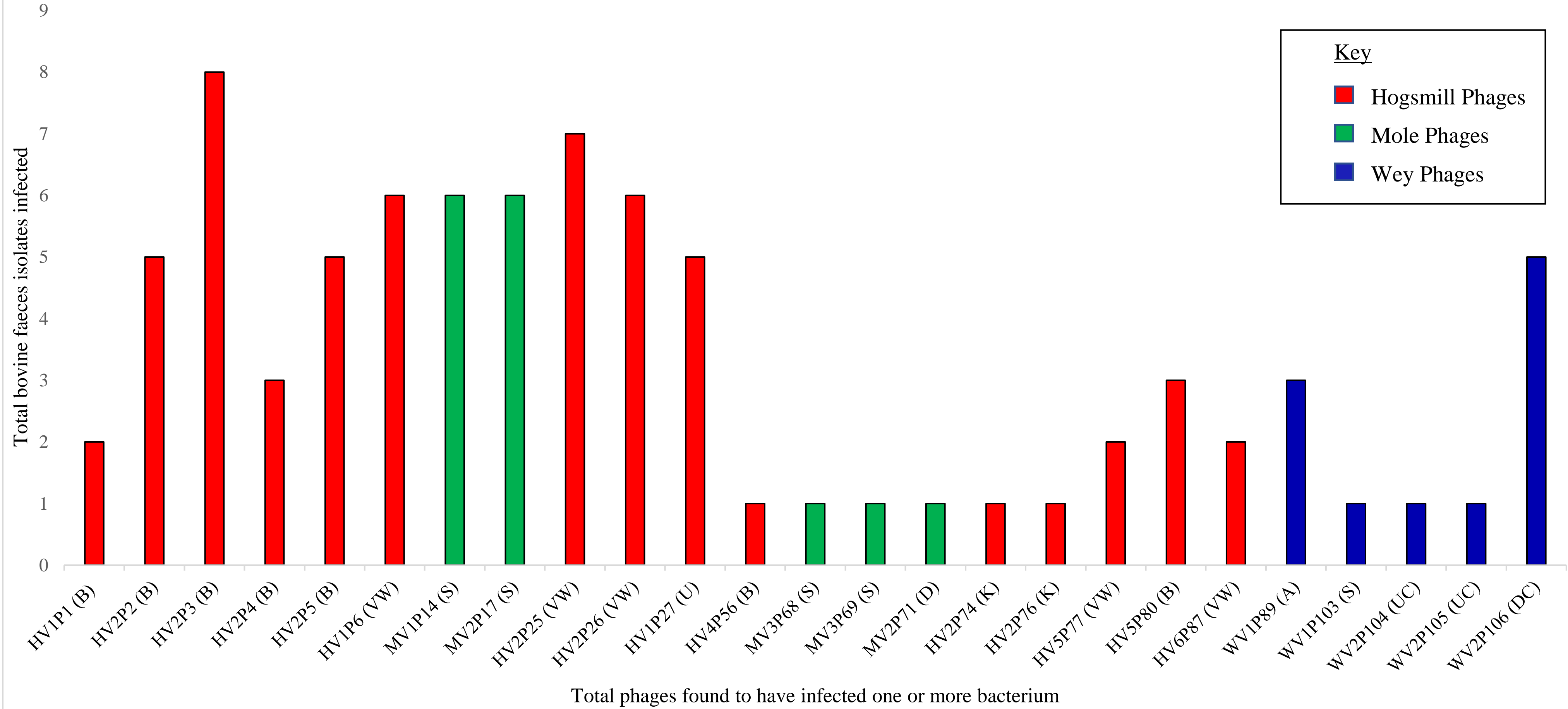


Figure 10. This data is from Table 7. It presents which phages are possibly broad ranged compared to the other phages found throughout the experiment. Different colours of the bars show the different locations in which the phages were initially isolated from.

Host range of initial phages against human isolates

Phages from Table 6 and 7 were then tested against human ESBL producing *E. coli* isolates. Figure 14 represents the data in Table 9. Phages HV2 P5 (B), HV3 P49 (B) and HV4 P53 (B) are examples of phages that had infected one strain besides their original target bacteria (Figure 11). Phage HV1 P6 (VW) infected six other bacterial *E. coli* strains and phage HV1 P43 (K) had infected five other bacterial strains. Both phage HV1 P47 (VW2) and HV2 P4 (B) had infected three other bacterial strains.

Out of the eight River Mole phages, only two phages had infected one other bacterial strain. Phage MV2 P16 (S) had infected three other bacterial strains. Phages MV1 P15 (S), MV2 P18 (S), MV2 P19 (S), MV2 P20 (S) and MV2 P23 (U) had infected two other bacterial strains. Lastly, Phage TV1 P19 shown in pink was the only phage isolated from River Thames that had infected one other bacterial strain. Figure 10 and Figure 11 show that phage HV1 P6 (VW) was the only phage that had infected twelve bovine faecal and human ESBL strains. Of the total twenty-seven bacteria used to determine the host range, twelve phages successfully infected three or more bovine faecal and human ESBL strains.

Out of the thirteen phages isolated from River Mole, three phages had infected three or more bovine faecal and human ESBL strains. Finally, out of the five phages isolated from River Wey to determine host range, only two phages infected three or more bacterial strains. Of the two hundred bacteria used to isolate phages and to determine the broad range, only thirty-five bacteria were susceptible. Out of the thirty-five bacteria used, *E. coli* 2P was infected by eleven phages, *E. coli* 6 was infected by six phages, *E. coli* 16P and *E. coli* 5P both were infected by seven phages. These bacteria were more susceptible to phage infection.

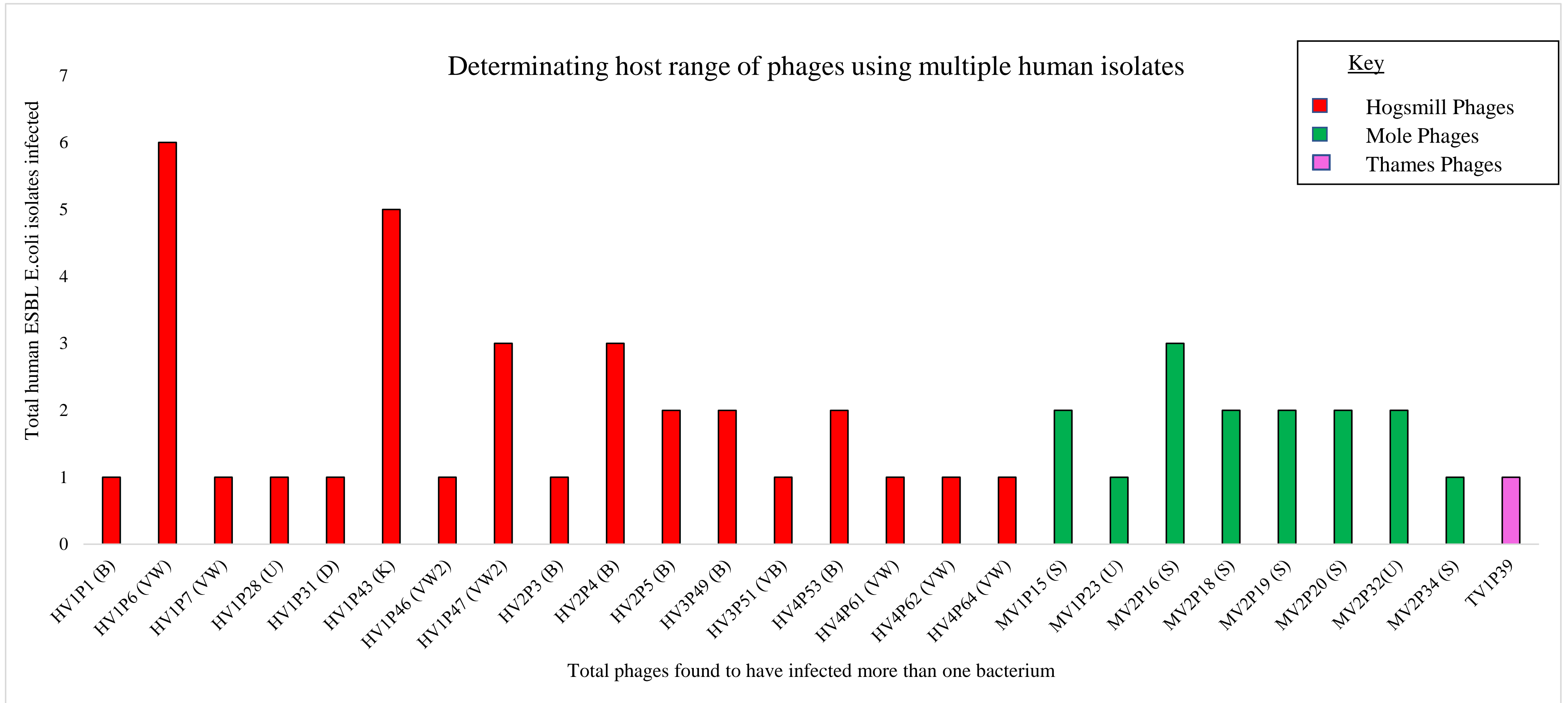


Figure 11. This data is from Table 8. It presents which phages are possibly broad ranged compared to the other phages found throughout the experiment. These phages were found to have infected human ESBL *E.coli* isolates.

Killing Titre of isolated phages

Using isolates from Table 1-4, phages were serially diluted to 10^{-8} pfu/ml as shown in Table 10 and 11 in Appendix B. Phages MV2 P33 (U), MV2 P20 (S), HV6 P77 (VW), HV1 P6 (VW), HV1 P43 (K) and HV4 P53 (B) only presented phage activity at 10^{-1} pfu/ml concentration. Phages HV2 P5 (B), HV4 P55 (B), MV2 P17 (S) and MV2 P71 (D) had killing titre concentrations of 10^{-6} pfu/ml which suggests that they are potent phages against the target bacteria. Phages WV1 P109 (G), WV1 P110 (AB), HV2 P74 (K) and HV2 P4 (B) have shown phage activity at 10^{-8} pfu/mL and still would be active at a dilution greater than 10^{-8} pfu/ml, which suggests that these phages were extremely potent. Tables 10 and 11 show a range of phage activity when diluted to 10^{-8} pfu/ml and show that fourteen phages from different visits and locations had killing titre concentrations of 10^{-3}pfu/ml.

MALDI-TOF analyses of isolates

The MALDI-TOF analysis was performed on thirty-four isolates from Table1-4, by Great Ormond Street Hospital (Dr Francis Yongblah). The data provided was used to create graphs that identified peaks at specific frequencies (*m/z*) with different intensities (Figures 12-15 (rest in Appendix C)). To verify the peaks on the graphs, a limit was added to avoid adding lower intensity values and potential signal noise. Therefore, intensity values greater than 4000 at specific frequency peaks were used for analysis. Frequencies (2320, 2340, 2360, 2370, 2390, 2400, 3790, 3800, 3810, 3820, 3830, 4360, 4860, 5090, 5750, 6250, 6310, 9060 and 9730 *m/z*) all have five or more bacteria with the same frequency, which suggests similarity within the *E. coli* isolates used. The isolates were then separated into their specific groups such as farm codes (ESBL, BIN / F2, DW and LV3). Within each group, specific peaks have been shown to further clarify the *E. coli* isolates used in this project. For example, in the DW group, isolates had peaks at both frequency 3790 and 6250 *m/z*, in the F2 group, all isolates had peaks

at 9730 *m/z*, in the BIN group both isolates had peaks at 2360, 3790, 5090 and 9730 *m/z*, in the LV3 group, both strains had peaks at 3790, 5090, 7060 and 9730 *m/z*. Another example can be shown in the ESBL group where *E. coli* isolates ESBL 11B, 7 and 8P have frequency peaks at 2320, 2400, 3790, 5090, and d 9730 *m/z* which suggests that they are similar in species. However, ESBL 7 and 8P have an additional specific peak at 4860 which is not shown in ESBL 11B. This suggests that these two isolates are similar at the strain level.

Isolates were then separated into groups containing similar frequency peaks (Tables 12-14 in Appendix C). For example, in Group 1, *E. coli* isolates ESBL (11P, 6, 7, 8P), BIN 23, F210D and F24D had frequency peaks at 2320, 2360 and 9730 *m/z*. Within this group, six out of seven isolates had similar peaks at 3790, 3810 and 3820 *m/z*, which could indicate further similarities within the different strains of that group. Together, these strains were used to isolate seventeen phages from all three rivers. Group 2 had *E. coli* isolates; ESBL 4P, Bin 23E, (DWV1 12A & 22B2) and F210D at frequency peaks of 2360, 3790 and 5690 *m/z*. Three out of five isolates had additional peaks at 3810, 3820 and 6250 *m/z* ad together had twenty phages that were isolated using these bacteria. Group 3 had *E.coli* isolates; ESBL (7P & 9), F2 KIA and F216B which had peaks at 2320, 2370, 2400, 3790 and 9730 *m/z*. Within this group, three out of four bacteria have peaks at 3430, 4360,5090,6250 and 9060 *m/z*. This suggests that compared to Group 1 & 2, the isolates of Group 3 showed greater similarities with their counterparts. Collectively, these four isolates have been used to isolate thirteen phages in this project, mostly from samples collected at Hogsmill river and River Mole. Group 4 had *E. coli* isolates; ESBL (2, 5P, 3P & 2P) at frequency peaks of 2370 and 5090 *m/z* but collectively had isolated twenty-one phages from samples collected at Hogsmill river and River Mole. Group 5 of *E. coli* isolates DWV1 (25D, 2B) and DWV2 9F had peaks at 2320, 2360, 3790 and 6250 *m/z*. Two out of the three isolates had additional peaks at 3820, 3830, 4360, 5090, 9060 and 9730 *m/z*. Fifteen phages were also isolates using these isolates. Lastly, group 6 of *E.*

coli isolates DWV1 26B2 and DVW2 20F had the most peaks of 2330, 3790, 4360, 5090, 6250, 6310 and 9060 *m/z* but only isolated five phages. The last four isolates ESBL 10P & 6P, LV3 10A and F2 8C had the least number of peaks at 5090, 6250 and 9730 *m/z* and collectively isolated sixteen phages.

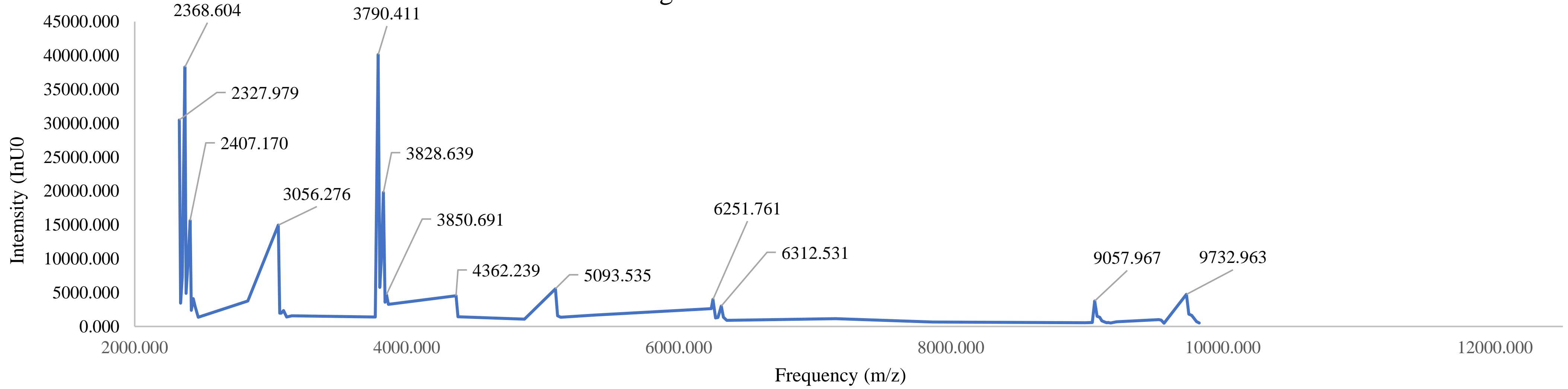


Figure 12 : E. coli ESBL 11B
Intemsity (InU0
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2368.604
2327.979
2407.170
3056.276
3790.411
3828.639
3850.691
4362.239
5093.535
6251.761
6312.531
9057.967
9732.963

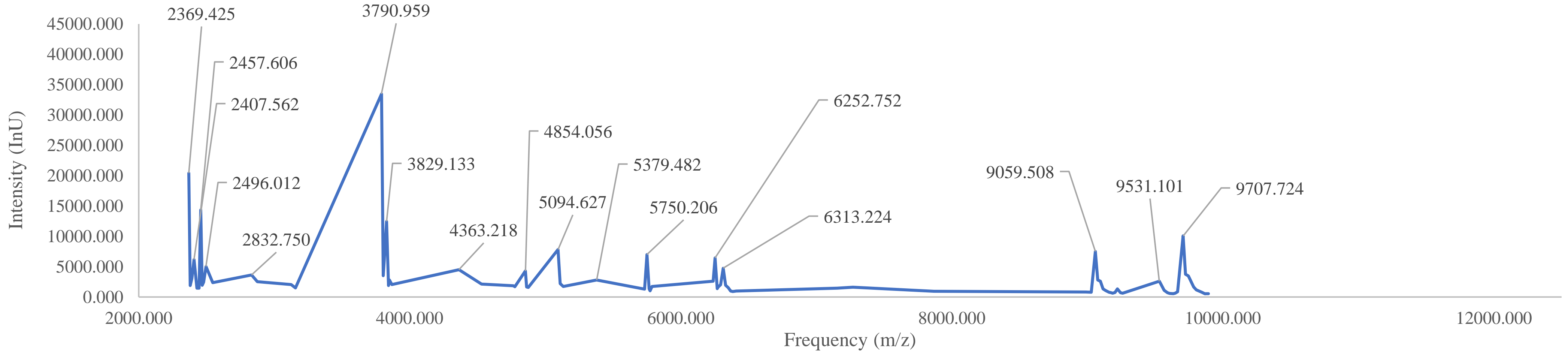
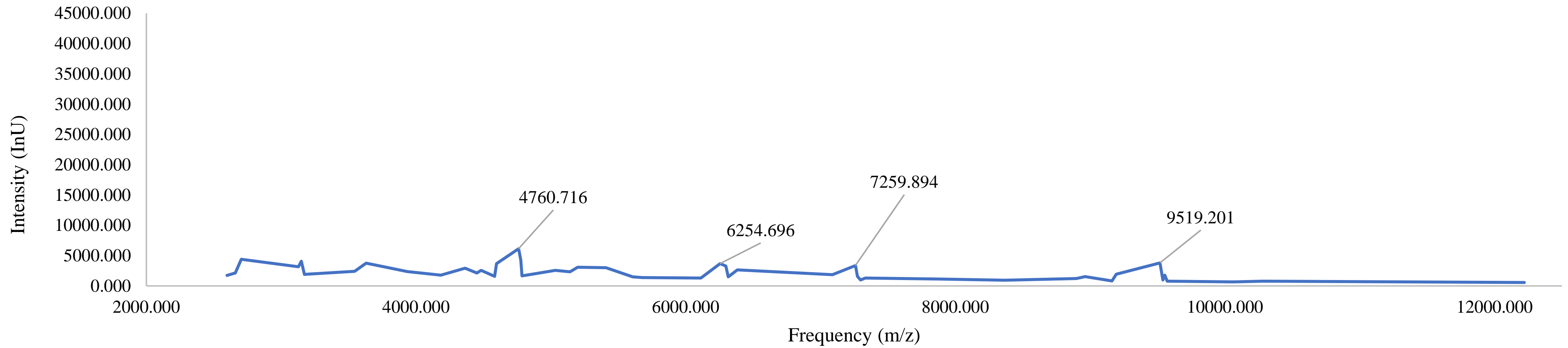


Figure 13: E. coli DW V1 2A
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2369.425
2457.606
2407.562
2496.012
2832.750
3790.959
3829.133
4363.218
4854.056
5094.627
5379.482
5750.206
6252.752
6313.224
9059.508
9531.101
9707.724



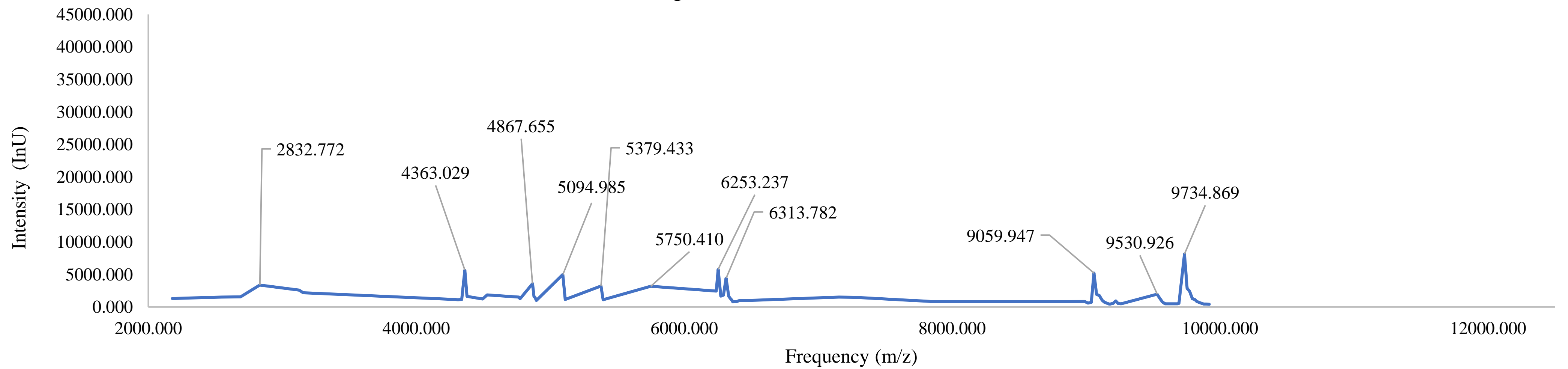


Figure 12-15: The data provided by Dr Yongblah was used to plot these graphs to analyse strain similarities and differences between the 34 isolates provided, rest of the graphs are in Appendix C.

DISCUSSION

OVERVIEW

In this study, a total of one hundred and eleven bacteriophages with different host specificities were shown to infect isolates of *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis* and *Klebsiella* sp. Bacteriophages were isolated using bovine faecal and human ESBL producing *E. coli* and *Klebsiella* sp isolates from the Kingston University culture collection. This study supports the existing theory that phages can kill many different species of bacteria, which could be utilised in agriculture, environment safety, food safety and treating humans with existing bacterial infections to eradicate bacterial populations (Wang *et al*., 2005; Abedon, 2011; Patey *et al.,* 2018; McCallin *et al*., 2019 and Shanin *et al*., 2019). The work here represents a starting point to show that phages are indeed present in the environment and active against antibiotic-resistant pathogens. This work might indicate the potential to harvest these phages as a possible therapeutic agent in the future to help in the battle against AMR.

In line with the article by Clokie and colleagues (2011), phages in this study were readily isolated from water samples that were collected from different rivers. This supports the suggestion seen in many articles used in the study that phages are ubiquitous in nature (Davies and Davies, 2010; Clokie *et al*., 2011; Jończyl *et al*.,2011; and Abedon, 2016). Out of the four sites, the Hogsmill river was frequently visited as it was within Kingston Upon Thames. During the pandemic, travel was limited by the government, therefore it was harder to obtain samples from other rivers and areas. Due to the higher number of samples taken, the Hogsmill river did present to have the most phages isolated in this study (As shown in Table 5) than the other sites.

This study also investigated the host specificity of the isolated phages from the different sites, in which eleven phages were shown to infect five or more (bovine and human) isolates. This suggested that these phages were broad ranged. Each sample site had at least one phage that had infected four or more bacterial strains and species. This study also demonstrated that there was no correlation between phage host specificity and killing titre, however, there were phages that were broad ranged with high killing titre which show promising results that can be used in phage cocktails in the future.

Different factors may contribute to successful infection.

The reports provided by the 'Environment Agency' in 2019 suggested that Hogsmill River, River Mole and River Wey failed to achieve a good status regarding their pollution levels (Environmental Agency, 2019). Bhetwal and colleagues (2017) suggest an interesting point that different factors such as pollution caused by the general public or by water industries can affect phage adaptations or host range. The article by Bhetwal and colleagues (2017) demonstrate the survival tactics of phages by adapting to the surrounding conditions to make them favourable for successful infection. This can be supported by the phages shown in Table 6-8, where certain phages from the same area of collection, infected one or more different bacteria that could appear in both humans and animals. However, this could be either a promiscuous phage where the same phage is infecting different bacteria or different phages infecting different bacteria.

Despite the different locations of the phages isolated, they were still found to have killed the targeted bacteria used in this study which were isolated from different sources. It was also concluded that there was no direct link between whether phages infected more bovine isolates near farmland or if phages infected more human isolates near wastewater treatments plants.

For example, water samples from location four were collected in an area with higher human activity. At this location of the Hogsmill River, twenty bovine isolates were susceptible to twenty-six phages isolated. Of these twenty-six phages, eight phages were found to have infected nine human ESBL-*E. coli* isolates. At location ten of River Mole, samples were collected in an area with open fields and less human activity, however, nine bovine isolates were susceptible to thirteen phages. Of those thirteen phages, six phages infected six human ESBL- *E. coli* isolates. Lastly, water samples from location twelve at River Wey were collected directly from a sewage outlet, however, only one bovine isolate was susceptible to nine phages and no ESBL- producing isolates were susceptible to these phages.

Although these locations had successful phage isolation using the target bacteria, some locations had less phage isolation. For example, location two at Hogsmill River was close to a wastewater treatment plant with higher human activity, however, only one phage targeted one bovine isolate and no human isolates were infected. Location eleven at River Mole was surrounded by open fields and close to a farm park, however only four bovine isolates were susceptible to four phages. No human isolates were susceptible to these phages. Lastly, location fifteen at River Wey was close to the local area and open fields, however, only one bovine isolate was susceptible to one phage. Phages isolated near wastewater treatments did not have a specific affinity towards human isolates and phages isolated near farmlands did not have a specific affinity towards bovine isolates. It could imply that these phages may be promiscuous or may have encountered a bacterium with a common binding site, or it may have successfully infected a particular target bacterium in the past. Although, it could also suggest that these phages had to adapt and evolve to survive. This data illustrates that in this study, there was no direct link between location and target isolates.

Environmental factors such as climate conditions, acidity, salinity, and ionic imbalance, can affect phage survival and contribute to morphological changes which may impact phage

survival. For future work, each factor could be tested individually to see how these factors affect phage infection. The vast adaptations of phages allow them to coexist with bacteria in different environments (Clokie *et al.*, 2011).

Hogsmill River and how the weather may have affected phage levels.

Table 5 exhibits the time and weather conditions on the day of water collections and three days before. The Autumn of 2019 was recorded to be one of the heaviest rainfall periods across the UK (Met Office, 2019). The data in Table 5 shows a difference in temperature throughout the autumn to winter months of 2019. At Hogsmill river, between October and November 2019, temperatures were 8-11ºC± 3ºC. Decreased seasonal sunlight, low temperatures and the time-of-day samples were collected contributed to 19 phages isolated at Hogsmill river during October and November 2019. This shows that nineteen phages had desirable conditions for successful infection, however, this is dependent on each phage and its adaptations. By December 2019 at Hogsmill River, temperatures were between 3-8 ºC. However, Table 5 shows that twenty-seven phages were isolated during December 2019 at the same locations. Again, this could suggest that the same phages were isolated or different phages were isolated and infected similar or different bacteria. By January-February 2020, temperatures increased to 11-12 ºC and only fifteen phages were isolated. Based on the data provided, it shows that the phages isolated at Hogsmill river in this specific study, thrive better in lower temperatures.

River Mole and how the weather affected phage levels.

Table 5 also shows the weather and temperature at River Mole, throughout November 2019- January 2020, where temperatures fluctuated between 8-10 ℃. Unlike phages isolated at Hogsmill river, only twenty-four phages were isolated in three months at River Mole and the site was visited three times. This can suggest that phages at this site did not respond to the bacteria used in this study, or there were fewer phages present or that bacteria had evolved to prevent infection from these phages.

_River Wey and how the weather affected phage levels.

By February 2020, temperatures at River Wey were between 3-4 ℃ and although this site was visited once, fifteen phages were isolated. However, in August 2020, temperatures significantly increased to 15-16 ℃ and only eight phages were isolated from six different sites.

In conclusion, at all three rivers, more phages were isolated during lower temperatures which seem to benefit phage infection. This also supports the facts presented by Bhetwal and colleagues (2017) that factors such as high temperature, high UV light and a lack of material getting into the river can decrease phage activity and infection. The presence of UV light can disinfect the water, which would result in less microbiological contamination in the water, hence disrupting the phage-bacteria cycle.

Coexistence of bacteria and phage and how bacteria affected phage activity.

Of the two hundred isolates, only thirty-five bacteria were susceptible to either one or more phages. This could imply that, as described in the article by Lin and colleagues (2017), phage and bacteria coexist. Either bacteria are evading phage infection, phages are not equipped to infect bacteria, or the bacteria were not present in the first place. It is valid to think that coexistence is present as it is stated in many other previous research papers such as in Clokie and colleagues (2011) and Oechlin and colleagues (2018). The coexistence of phage and bacteria, balance the microbial community in different environments. The results in this study show that not all phages isolated, infected all bacteria present. This also supports the idea that phages have different morphological structures or defence systems to overcome different strains or species of bacteria (Dimmock *et al*., 2007, Bhetwal *et al*., 2017).

Host specificity and how the host range was established.

Throughout this study, samples were tested against *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis* and *Klebsiella* sp. The study demonstrates the different host specificity of phages isolated from different rivers (Figure 10 & 11). Valid methods mentioned in previous studies such as in (Kutter *et al*., 2005 and Ross *et al*., 2016) demonstrated that spot and plaque assay was successful in this study. It must be mentioned that it is not known if the phages and the bacteria are different as no genetic typing was able to be performed. This would be useful to carry out in future research.

A study carried out by Wang and colleagues (2005), isolated phage Ø9882 from hospital sewage, which infected eleven ESBL-*E.coli* strains. The results in this current study also show similar findings carried out by Wang and colleagues (2005). For example, in this current study, six phages could be considered to be broad ranged, as they infected more than four isolates.

For example, phage HV1 P6 (VW) infected twelve different strains of bacteria (five bovine *E. coli* and one *P. vulgaris* bovine isolates and six human ESBL *E. coli* isolates). Phage HV2 P3 (B) infected nine bacteria, (seven bovine *E. coli* isolates and one *P.vulgaris* bovine isolate and one human ESBL *E.coli* isolate). Another phage, HV2 P25 (VW), infected seven different bacteria (five bovine *E. coli* isolates and two *P. vulgaris* bovine isolates). Phage HV2 P5 (B) infected seven different bacteria (four bovine *E. coli* isolates, one *P. vulgaris* bovine isolate and two ESBL *E. coli* isolates). Other phages such as HV2 P26 (VW), MV1 P14, MV2 P17 (S) infected six bovine bacteria (five *E. coli* isolates, one *P.vulgaris* isolate). Phage HV2 P4 (B) infected six bacteria (two bovine *E. coli* isolates, one *P.vulgaris* bovine faecal isolate and three ESBL- *E. coli* isolates) The results indicate that ESBL- producing bacteria (which are potentially hard to treat with existing antibiotics) can also be killed by phages readily found in environmental settings. Other phages that might be considered as broad range are phage HV2 P2(B) infected five bovine faecal bacteria (four *E. coli* and one *P.vulgaris* isolate), phage HV1 P27 (U) infected five bovine faecal bacteria (three *E. coli* and two *P.vulgaris* isolates), phage WV2 P106 (DC) infected five bovine faecal bacteria. These eleven phages might be considered as broad range phages which can be used in combination with other phages as a cocktail in the future.

Each sample site had at least one phage that had infected four or more bacterial strains and species (bovine or human isolates). This observation is very promising as Carillo and Abedon (2011) mention, that broad range phages are desirable in phage therapy. The idea to have multiple phages in a single cocktail to target many different bacterial strains and species is more desirable than using a single phage that infects one specific bacterium. However, the disadvantage of creating a phage cocktail is that it could be a costly and time-consuming process, depending on the multiple phages added. These phages could be manipulated to adapt

to infect multiple species of bacteria, without introducing virulent factors into the bacterial DNA that could lead to phage resistance (Carillo and Abedon,2011).

How killing titre was determined for the isolated phages

Killing titre was used to determine the lowest viral concentration that was still active against a target bacterium. This was done to demonstrate how potent a phage was when it was diluted. Spot assays were used to determine killing titre. Plaque assays were also used, however certain phages did not produce plaques and therefore reverted to spot assays. From this study, the information in Tables 10 and 11 show different concentrations of killing titre, which could suggest a variation in the phages isolated. The range of concentrations could also suggest that not all phages are the same in species and efficacy (Clokie *et al*., 2011). Phages HV2 P4 (B), HV2 P74 (K), WV1 P109 (G), WV1 P110 (AB) had killing titres of 10^{-8} pfu/ml. These phages would also be active at concentrations greater than 10^{-8} pfu/ml, which suggests that they are very potent at diluted concentrations. Phage HV2 P4 (B) was also considered to be broad-ranged, as it infected three different strains of *E. coli*, *P. vulgaris* bovine isolates and three different human ESBL-*E. coli* isolates. However, phage HV2 P74 (K) would be considered as a narrow range phage as it infected two different *E. coli* bovine isolates and both WV1 P109 (G) and WV1 P110 (AB) only infected one bacteria. Phages HV2 P5 (B), HV4 P55 (B), MV2 P17 (S) and MV2 P71 (D) had killing titre concentrations of 10^{-6} pfu/ml, which suggests that they are also potent phages against the target bacteria. From this current study, phage HV2 P4 (B), phage HV2 P5 (B) and MV2 P17 (S) are also considered to be broad range as they infected 5 or more different bacteria including ESBL- producing isolates. These three phages would be considered as broad-ranged phages that are potent against a range of bacteria

which could be highly desirable for possible application in the Agro-food sector or phage therapy in humans.

Tables 10 & 11 show no correlation between the killing titre and host range, as it illustrates that there is high variability within the phages isolated in this study. These phages show that it is not essential to have a high killing titre and to be broadly ranged. Perhaps, when phage therapy is accepted. some alternative therapies may need to use one highly specific narrow range phage with a high killing titre to target a specific bacterium, instead of a phage cocktail targeting multiple bacteria. It is assuring that phages with different killing titres and host ranges can be readily found in nature. For example, in this study, some phages such as HV1 P6 (VW) and HV1 P43 (K) have low killing titre but are broad range phages. Other phages: HV1 P28 (U), HV2 P74 (K) and WV2 P104 (UC) have high killing titre concentrations but are narrow range phages, only infecting one bacterium. Phage HV1P31 (D) is an example of a phage having a low killing titre and a narrow range. It must be noted that phages with a lower killing titre could affect the overall titre of the cocktail as one might have to increase it by adding a phage with a higher titre. However, there are a few phages in this study that have killing titres between 10^{-5} to 10^{-8} pfu/ml that are broad ranged as mentioned above. Overall, Hogsmill river and River Wey showed a variety of killing titres and host ranges.

Throughout this experiment, the killing titre method did not work on some phages. For example, phage HV1 P43 (K) and HV4 P53 (B) did not work when the plaque assay method was applied. When testing these phages, the plaque formation was not visible, which could mean that the viral concentration was either too potent, a lower aliquot should have been used or further dilution was needed. Despite this issue, it did work when the spot assay was carried out. During the spot assay, five 'zones of clearing' were visible within the plate, which show phage presence. Another example of the spot assay working rather than the plaque method was when phage HV1 P27 (U) was tested twice (in 4 months) using the same bovine isolate *E. coli*

F2 8C and the first result had a concentration of 10^{-2} pfu/ml and then 10^{-4} pfu/ml. This might have been a user error as certain phages mentioned above were viable when the spot assay method. The phages either did not survive the freezing and thawing process. Overall, this shows that different phages have a variety of killing titres, host ranges and lifespans.

How MALDI-TOF can be used to show phage specificity

The MALDI-TOF procedure was conducted by Dr Francis Yongblah at Great Ormond Street Hospital using thirty-four *E.coli* isolates from Table 1-4. The frequency (*m/z)* and intensity values (InU) were used to plot a graph to visualise specific proteins found in each isolate. The peaks at specific frequencies were used to distinguish similarities within each isolate (Calvano *et al.,* 2016). Once similarities within strains were identified, it can be stated that phages found to have infected one isolate can also infect an isolate with similar protein biomarkers. Of the thirty-four isolates, most were found to have peaks at specific frequencies, which were then classed as clusters. Lynn and colleagues (1999) state that the Family *Enterobacteriaceae* have five specific peaks at 4364, 5380, 6384,6856 and 9540 *m/z* which indicates that *E. coli* species should indicate frequency peaks within the range stated. This project shows that most *E. coli* isolates are at least in the range of three out of the five specific peaks of *Enterobacteriaceae.*

Isolates were separated into nine groups based on the similar number of specific peaks found in each isolate (Table 12-14 in Appendix C). Out of all the groups, Group 4 had four *E. coli* ESBL isolates which were used throughout this project to isolate twenty-one potentially distinct phages. This could suggest this group was more susceptible to phage infection compared to the other isolates, for example, *E. coli* LV3 10A was only susceptible to two phages isolated at Hogsmill River. However, Group 1, 2, 3 and 5 had thirteen- seventeen phages collectively, which could also assume that bacteria of similar DNA structure/protein

biomarkers peaking at similar frequencies of these groups could be highly susceptible to phage infection.

Previously, frequency values were compared against each isolated to show similarities and differences, however, the data also shows intensity values detected at the same frequency peak of isolates can be analysed. For example, at peak 2400 *m/z*, *E. coli* isolate ESBL 9 has an intensity of 5338.38 InU, however ESBL 4P although of the same strain, had 4303.89 InU. This shows that although these ESBL strains have a protein at 2400 *m/z*, they have different intensity values, which could suggest further differences within the strain. If it is compared to *E. coli* F2 K1A, at the same frequency peak, it has a closer value of 5529.03 InU, which could suggest that ESBL 9 and *E. coli* F2 K1A are closely related to each other than ESBL 4P.

Once isolates were grouped by similarity of peaks present (Groups 1-9), phages that were isolated throughout this study using the thirty-four isolates were listed to see which phages were common in each group. If a certain phage was common in three or more groups, it was classed as broad-ranged as it could infect different bacteria. For example, phages HV2 P5 (B), HV1 P6 (VW), MV2 P16 (S), and HV4 P53 (B) were found in four groups. This indicates that the strains in these groups were highly susceptible to the phages in this study. Secondly, phages HV2 P3 (B), HV2 P4 (B), HV1 P27 (U), MV2 P32 (U), HV1 P43 (K) and HV5 P62 (VW) were only common in two groups. This information can then be compared to the experiments previously done in this study. Phages HV2 P3 (B), HV2 P4 (B), HV5 (B) and HV1 P6 (VW) were shown to have qualities of broad range phages by infecting six or more bovine or human ESBL isolates. It is important to understand that these phages could be effective at tackling potentially problematic (resistant and/or pathogenic) organisms. These broad range phages could be added to a cocktail and used effectively to target multiple ranges of organisms in future experiments.

Storage of phages, how phages were later affected after defrosted and limitations.

Due to the pandemic, samples were stored at (-80ºC) and defrosted at least three times, due to lab closure. This could be the reason why some defrosted phages were not active. Although a few phage supernatants were stored at 4°C for a maximum of six days, this did not appear to harm the activity of the phage. It also shows evidence of the possibility of biological variance between phage strains. For example, some phages had faded zones with minimum activity, while others such as MV2 P70 (D) and MV2 P71 (D) wiped out the plate. This might suggest that different phages have a different survival time in storage or storage requirements.

The three national lockdowns due to the COVID-19 pandemic affected the current study in several ways. Firstly, the number of times individual river sources were tested, due to its location close to the university and its ease of access, the Hogsmill river was tested the most. Secondly, samples were not collected in summer, which could have given an insight into how weather conditions affected phage activity. Thirdly, phages were all stored the same, however because of the multiple national lockdowns, interrupting the flow of the study, some phages did not survive the constant thawing, growing and freezing cycle because of the different lifespans. Lastly, although the viral genomic analysis was considered, it was beyond the scope of this study to have imaged the phages isolated for classification, due to its complex method and no extra time available for troubleshooting. Pulsed-Field Gel Electrophoresis was unsuccessful in this project due to troubleshooting issues and no available time, MALDI-TOF was performed in its place. Obtaining results were delayed and if all isolates were given to analyse a greater result would have been shown, however it was not possible due to the current pandemic issues.

CONCLUSION

In conclusion, phages can be readily isolated from the environment and were shown to have killing activity against a range of different bacteria, including human ESBL-producing *E. coli* isolates, which are possibly harder to kill using existing antibiotics. Phages although discovered before antibiotics, have the potential to be utilised in the fight against AMR. Many research articles and studies such as Wang *et al*., 2005, Clokie *et al*., 2011, Bhetwal *et al*.,2017, Fernàndez *et al.,* 2018 and Rodhe *et al.,* 2018 discuss and demonstrate different aspects of phages, which could be extremely valuable in the search for alternative agents to combat AMR. Phage research could fulfil the interesting points made by Lord O'Neill in terms of looking for alternatives to a conventional antibiotic to treat AMR organisms. However, further research is needed to establish if phage therapy can be approved by regulatory agencies for use in different fields such as environmental safety, food safety or treating human bacterial infections.

Recommendations and further work

To carry this current study on, the viral genetic analysis would be extremely helpful in classifying phages isolated using river samples. Electron microscopy could produce an image of the phages found to assess and analyse different morphological structures. If these series of experiments were to be successful, a phage cocktail could be tested on different surfaces (such as in hospitals) to see if it does successfully kill the antibiotic-resistant bacteria present, however, this would still be in the works. Phage therapy is not currently fully accepted, and more research is needed, however, this current study has shown promising results in terms of the potential of phages to help combat antibiotic-resistant bacteria and helping to combat AMR in the future.

APPENDIX A

List of equipment used throughout the project and Table A.

- Plastic Agar petri plates
- Automatic dispenser (10ml)
- Automatic pipette (2-1000µl)
- 0.5 M0.5 MacFarland Standard solution
- Bunsen Burner
- Sterile 50ml Falcon Tubes
- Sterile cotton buds
- Sterile inoculating loops
- Centrifuge machine set for 10 mins at (1448 x g) 3000 rpm
- Millipore 0.2µm cartridge filter
- Disposable syringe (10ml)
- Tweezers
- Plastic bijoux bottles (7ml)
- 96-well plates

Table A:

Instructions on how to make given solutions used throughout this study

Solutions	**Directions**
Nutrient Agar (NA)	Dissolve 14g of NA in 500ml of distilled water in a glass bottle and autoclave
Nutrient Broth (NB)	Dissolve 7.5g of NB in 500ml of distilled water. Decant to 5ml glass bottles and autoclave
Ringer's Solution (RS)	Dissolve 1 tablet in 500ml of distilled water. Decant to 5ml glass bottles and autoclave

APPENDIX B – *TABLES CREATED IN THIS STUDY*

Table 1: Bacteria isolated using bovine faeces from 'Binnington' farm		
Bacteria Species	**Farm code**	**Visit number**
E. coli	BIN 219C	1
E. coli	BIN 23E	1
E. coli	BIN 24E	1
E. coli	BIN 312B	1
E. coli	F2 1E	2
E. coli	F2 2G	2
E. coli	F2 4D	2
E. coli	F2 7A	2
E. coli	F2 8C	2
E. coli	F2 9A	2
E. coli	F2 10D	2
E. coli	F2 16B	2
E. coli	F2 K1A	2
E. coli	BV3 20C	3
E. coli	BV3 21C1	3
E. coli	BV3 23	3
E. coli	BV3 23C	3
E. coli	BV3 23D	3
E. coli	BV3 24D	3
E. coli	BV4 18C	4
E. coli	BV4 18D	4
E. coli	BV4 19B	4
E. coli	BV4 20B	4
E. coli	BV4 21C	4
P. vulgaris	F2 1B	2
P. vulgaris	F2 4C	2
P. vulgaris	F2 4E	2
P. vulgaris	F2 10C	2
P. vulgaris	F2 15A	2
P. vulgaris	F2 16A	2
P. vulgaris	BV3 10b	3
P. vulgaris	BV3 10B	3
P. vulgaris	BV3 18d	3
P. vulgaris	BV3 21b1	3
P. vulgaris	BVT4 20C	4
P. vulgaris	BV4 21B	4
P. vulgaris	BV4 23B	4
P. aeruginosa	F2 3F	2
P. aeruginosa	F2 5C	2
P. aeruginosa	BV3 2b	3
P. aeruginosa	BV3 2C	3

P. aeruginosa	BV3 3C	3
P. aeruginosa	BV3 3e	3
P. aeruginosa	BV3 4F	3
P. aeruginosa	BV4 2b	4
P. mirabilis	BV3 20B	3
P. mirabilis	BV4 11B	4
P. mirabilis	BV4 15D	4
Klebsiella sp	F2 3G	2
Klebsiella sp	F2 4B	2
Klebsiella sp	BV3 3A1	3
Klebsiella sp	BV3 16C	3
Klebsiella sp	BV3 18B	3

Table 2:
Bacteria isolated using bovine faeces from 'Dan Williams' Farm

Bacteria Species	Farm code	Visit number:
E. coli	DWV1 2A	1
E. coli	DWV1 6A21	1
E. coli	DWV1 10d	1
E. coli	DWV1 11F1	1
E. coli	DWV1 122B	1
E. coli	DWV1 12B	1
E. coli	DWV1 16A1	1
E. coli	DWV1 16B	1
E. coli	DWV1 18B	1
E. coli	DWV1 18c	1
E. coli	DWV1 18d	1
E. coli	DWV1 19A	1
E. coli	DWV1 19C	1
E. coli	DWV1 20A1	1
E. coli	DWV1 21C	1
E. coli	DWV1 21D	1
E. coli	DWV1 21E	1
E. coli	DWV1 226	1
E. coli	DWV1 22B2	1
E. coli	DWV1 22C	1
E. coli	DWV1 22E	1
E. coli	DWV1 23D	1
E. coli	DWV1 23DE	1
E. coli	DWV1 24A12	1
E. coli	DWV1 24C1	1
E. coli	DWV1 24C2	1
E. coli	DWV1 24D1	1
E. coli	DWV1 24D2	1
E. coli	DWV1 25B	1

E. coli	DWV1 25C	1
E. coli	DWV1 25D	1
E. coli	DWV1 25E	1
E. coli	DWV1 26B1	1
E. coli	DWV1 26B2	1
E. coli	DWV1 26D12	1
E. coli	DWV1 27A1	1
E. coli	DWV2 1A	2
E. coli	DWV2 2A	2
E. coli	DWV2 2C	2
E. coli	DWV2 3B1	2
E. coli	DWV2 3B2	2
E. coli	DWV2 3C	2
E. coli	DWV2 3F	2
E. coli	DWV2 4C2	2
E. coli	DWV2 4e	2
E. coli	DWV2 5e	2
E. coli	DWV2 6B2	2
E. coli	DWV2 6c	2
E. coli	DWV2 6d	2
E. coli	DWV2 7c	2
E. coli	DWV2 7d1	2
E. coli	DWV2 8d	2
E. coli	DWV2 9d	2
E. coli	DWV2 9e	2
E. coli	DWV2 9F	2
E. coli	DWV2 10d	2
E. coli	DWV2 10C2	2
E. coli	DWV2 11B1	2
E. coli	DWV2 11C	2
E. coli	DWV2 11d	2
E. coli	DWV2 11e	2
E. coli	DWV2 12d	2
E. coli	DWV2 12e	2
E. coli	DWV2 12F	2
E. coli	DWV2 13e	2
E. coli	DWV2 13F	2
E. coli	DWV2 14B	2
E. coli	DWV2 14C	2
E. coli	DWV2 14d	2
E. coli	DWV2 15B	2
E. coli	DWV2 15d	2
E. coli	DWV2 25C	2
P. vulgaris	DWV1 11A	1
P. vulgaris	DWV1 17B1	1
P. vulgaris	DWV1 17B21	1
P. vulgaris	DWV1 23A2	1
P. vulgaris	DWV1 24A2	1
P. vulgaris	DWV1 24ED	1

P. vulgaris	DWV1 26A12	1
P. vulgaris	DWV2 4B1	2
P. vulgaris	DW2 7d2	2
P. aeruginosa	DWV1 8E	1
P. aeruginosa	DWV1 13F	1
P. aeruginosa	DWV1 22D	1
P. aeruginosa	DWV1 24ED	1
P. mirabilis	DWV1 20A2	1
P. mirabilis	DWV2 1d1	2
P. mirabilis	DWV2 4d	2
P. mirabilis	DWV2 6B1	2
P. mirabilis	DWV2 13C	2
P. mirabilis	DWV2 20E	2
Klebsiella sp	DWV1 19B	1
Klebsiella sp	DWV1 26A12	1

Table 3:
Bacteria isolated using bovine faeces from 'Lynwoods' Farm

Bacteria Species	Farm code	Visit number
E. coli	LV1 116A	1
E. coli	LV2 1e	2
E. coli	LV2 7B	2
E. coli	LV2 11e	2
E. coli	LV2 13c	2
E. coli	LV2 15C	2
E. coli	LV3 1D1	3
E. coli	LV3 6C	3
E. coli	LV3 10A	3
E. coli	LV3 16E	3
E. coli	LV3 21B	3
E. coli	LV3 25B	3
P. vulgaris	LV3 7e	3
P. vulgaris	LV3 10d	3
P. vulgaris	LV3 11b	3
P. vulgaris	LV3 19b	3
P. vulgaris	LV3 22C	3
P. vulgaris	LV3 23b	3
P. vulgaris	LV3 27b	3
P. aeruginosa	LV3 21C	3
P. mirabilis	LV3 2B	3
P. mirabilis	LV3 19C	3

Table 4: ESBL- producing bacteria isolated from human samples	
E. coli	*Klebsiella* sp
1P	11P
2	12
2P	13
3P	13P
4P	14
5	14P
5P	15
6	15P
6P	16
7	16P
7P	17P
8	18P
8P	19P
9	20P
10P	21P
11B	

Table 5: Initial phage isolation date and times								
Name of River	**Phage Code**	**Sample date**	**Time sample taken**	**Temp (°C)**	**Water temp (°C)**	**Weather on the day**	**Weather 3 days before**	**Location**
Hogsmill River	P1 HV1 (B)	15/10/2019	10:00	8	10	Cloudy and partly sunny	Scattered clouds	Blue Bridge
	P2 HV2 (B)	22/10/2019	10:00	10	11	Slight Fog	Cloudy and partly sunny	Blue Bridge
	P3 HV2 (B)	22/10/2019	10:00	10	11	Slight Fog	Cloudy and partly sunny	Blue Bridge
	P4 HV2 (B)	22/10/2019	10:00	10	11	Slight Fog	Cloudy and partly sunny	Blue Bridge
	P5 HV2 (B)	22/10/2019	10:00	10	11	Slight Fog	Cloudy and partly sunny	Blue Bridge
	P6 HV1 (VW)	22/10/2019	10:10	10	11	Slight Fog	Cloudy and partly sunny	Valley Walk
	P7 HV1 (VW)	22/10/2019	10:10	10	11	Slight Fog	Cloudy and partly sunny	Valley Walk
	P8 HV1 (VW)	22/10/2019	10:10	10	11	Slight Fog	Cloudy and partly sunny	Valley Walk
	P9 HV1 (VW)	22/10/2019	10:10	10	11	Slight Fog	Cloudy and partly sunny	Valley Walk
	P10 HV1 (VW)	22/10/2019	10:10	10	11	Slight Fog	Cloudy and partly sunny	Valley Walk
	P11 HV1 (VW)	22/10/2019	10:10	10	11	Slight Fog	Cloudy and partly sunny	Valley Walk
	P24 HV2 (VW)	25/11/2019	10:00	9	10	Drizzle and Partly Sunny	Partly Cloudy	Valley Walk
	P25 HV2 (VW)	25/11/2019	10:00	9	10	Drizzle and Partly Sunny	Partly Cloudy	Valley Walk
	P26 HV2 (VW)	25/11/2019	10:00	9	10	Drizzle and Partly Sunny	Partly Cloudy	Valley Walk
	P27 HV1 (U)	27/11/2019	09:20	10	10	Scattered Showers	Light Rain and Partly Cloudy	Upstream

	P28 HV1 (U)	27/11/2019	09:20	10	10	Scattered Showers	Light Rain and Partly Cloudy	Upstream
	P29 HV1 (P)	27/11/2019	09:26	10	10	Light Rain and Partly Sunny	Light Rain and Partly Cloudy	Post Office
	P30 HV1 (D)	27/11/2019	09:31	10	11	Light Rain and Partly Sunny	Light Rain and Partly Cloudy	Downstream
	P31 HV1 (D)	27/11/2019	09:31	10	11	Light Rain and Partly Sunny	Light Rain and Partly Cloudy	Downstream
	P41 HV1 (R)	03/12/2019	08:02	3	3	Clear Skies	Partly Sunny and light rain	Rose Theatre
	P42 HV1 (R)	03/12/2019	08:02	3	3	Clear Skies	Partly Sunny	Rose Theatre
	P43 HV1 (K)	03/12/2019	08:12	3	3	Clear Skies	Partly Sunny	Kingston College
	P44 HV1 (K)	03/12/2019	08:12	3	3	Clear Skies	Partly Sunny	Kingston College
	P45 HV1 (K)	03/12/2019	08:12	3	3	Clear Skies	Partly Sunny	Kingston College
	P46 HV3 (VW)	03/12/2019	08:21	3	3	Clear Skies	Partly Sunny	Valley Walk
	P47 HV3 (VW)	03/12/2019	08:21	3	3	Clear Skies	Partly Sunny	Valley Walk
	P48 HV3 (B)	03/12/2019	10:00	6	7	Sunny	Sunny	Blue Bridge
	P49 HV3 (B)	03/12/2019	10:00	6	7	Sunny	Sunny	Blue Bridge
	P50 HV3 (B)	03/12/2019	10:00	6	7	Sunny	Sunny	Blue Bridge
	P51 HV4 (VW)	03/12/2019	10:06	6	7	Sunny	Sunny	Valley Walk
	P52 HV4 (VW)	03/12/2019	10:06	6	9	Sunny	Sunny	Valley Walk
	P53 HV4 (B)	16/12/2019	09:19	8	9	Partly Sunny	Sunny and Partly Cloudy	Blue Bridge
	P54 HV4 (B)	16/12/2019	09:19	8	9	Partly Sunny	Sunny and Partly Cloudy	Blue Bridge
	P55 HV4 (B)	16/12/2019	09:19	8	9	Partly Sunny	Sunny and Partly Cloudy	Blue Bridge

	P56 HV4 (B)	16/12/2019	09:19	8	9	Partly Sunny	Sunny and Partly Cloudy	Blue Bridge
	P57 HV4 (B)	16/12/2019	09:19	8	9	Partly Sunny	Sunny and Partly Cloudy	Blue Bridge
	P58 HV4 (B)	16/12/2019	09:19	8	9	Partly Sunny	Sunny and Partly Cloudy	Blue Bridge
	P59 HV4 (B)	16/12/2019	09:19	8	9	Partly Sunny	Sunny and Partly Cloudy	Blue Bridge
	P60 HV4 (B)	16/12/2019	09:19	8	9	Partly Sunny	Sunny and Partly Cloudy	Blue Bridge
	P61 HV5 (VW)	16/12/2019	09:22	8	9	Partly Sunny	Sunny and Partly Cloudy	Valley Walk
	P62 HV5 (VW)	16/12/2019	09:22	8	9	Partly Sunny	Sunny and Partly Cloudy	Valley Walk
	P63 HV5 (VW)	16/12/2019	09:22	8	9	Partly Sunny	Sunny and Partly Cloudy	Valley Walk
	P64 HV5 (VW)	16/12/2019	09:22	8	9	Partly Sunny	Sunny and Partly Cloudy	Valley Walk
	P65 HV5 (VW)	16/12/2019	09:22	8	9	Partly Sunny	Sunny and Partly Cloudy	Valley Walk
	P66 HV5 (VW)	16/12/2019	09:22	8	9	Partly Sunny	Sunny and Partly Cloudy	Valley Walk
	P67 HV5 (VW)	16/12/2019	09:22	8	9	Partly Sunny	Sunny and Partly Cloudy	Valley Walk
	P74 HV2 (K)	14/01/2020	10:00	10	11	Light Rain	Partly Sunny and Cloudy	Kingston College
	P75 HV2 (K)	14/01/2020	10:00	10	11	Light Rain	Partly Sunny and Cloudy	Kingston College
	P76 HV2 (K)	14/01/2020	10:00	10	11	Light Rain	Partly Sunny and Cloudy	Kingston College
	P77 HV6 (VW)	14/01/2020	10:10	10	11	Light Rain	Partly Sunny and Cloudy	Valley Walk
	P78 HV5 (B)	25/02/2020	11:35	10	12	Partly Sunny	Light Rain and Cloudy	Blue Bridge

	P79 HV5 (B)	25/02/2020	11:35	10	12	Partly Sunny	Light Rain and Cloudy	Blue Bridge
	P80 HV5 (B)	25/02/2020	11:35	10	12	Partly Sunny	Light Rain and Cloudy	Blue Bridge
	P81 HV5(B)	25/02/2020	11:35	10	12	Partly Sunny	Light Rain and Cloudy	Blue Bridge
	P82 HV5 (B)	25/02/2020	11:35	10	12	Partly Sunny	Light Rain and Cloudy	Blue Bridge
	P83 HV7 (VW)	25/02/2020	11:41	10	11	Partly Sunny	Light Rain and Cloudy	Valley Walk
	P84 HV7 (VW)	25/02/2020	11:41	10	11	Partly Sunny	Light Rain and Cloudy	Valley Walk
	P85 HV7 (VW)	25/02/2020	11:41	10	11	Partly Sunny	Light Rain and Cloudy	Valley Walk
	P86 HV7 (VW)	25/02/2020	11:41	10	11	Partly Sunny	Light Rain and Cloudy	Valley Walk
	P87 HV7 (VW)	25/02/2020	11:41	10	11	Partly Sunny	Light Rain and Cloudy	Valley Walk
	P88 HV7 (VW)	25/02/2020	11:41	10	11	Partly Sunny	Light Rain and Cloudy	Valley Walk
River Mole	P12 MV1 (S)	11/11/2019	Collected by supervisor	8	N/A	Passing Clouds	Passing Clouds	Stepping Stones
	P13 MV1 (S)	11/11/2019	Collected by supervisor	8	N/A	Passing Clouds	Passing Clouds	Stepping Stones
	P14 MV1 (S)	11/11/2019	Collected by supervisor	8	N/A	Passing Clouds	Passing Clouds	Stepping Stones
	P15 MV1 (S)	11/11/2019	Collected by supervisor	8	N/A	Passing Clouds	Passing Clouds	Stepping Stones
	P16 MV2 (S)	17/11/2019	Collected by supervisor	7	N/A	Partly Cloudy	Passing Clouds	Stepping Stones
	P17 MV2 (S)	17/11/2019	Collected by supervisor	7	N/A	Partly Cloudy	Passing Clouds	Stepping Stones
	P18 MV2 (S)	17/11/2019	Collected by supervisor	7	N/A	Partly Cloudy	Passing Clouds	Stepping Stones

	P19 MV2 (S)	17/11/2019	Collected by supervisor	7	N/A	Partly Cloudy	Passing Clouds	Stepping Stones
	P20 MV2 (S)	17/11/2019	Collected by supervisor	7	N/A	Partly Cloudy	Passing Clouds	Stepping Stones
	P21 MV1 (U)	17/11/2019	Collected by supervisor	7	N/A	Partly Cloudy	Passing Clouds	Upstream
	P22 MV1 (U)	17/11/2019	Collected by supervisor	7	N/A	Partly Cloudy	Passing Clouds	Upstream
	P23 MV1 (U)	17/11/2019	Collected by supervisor	7	N/A	Partly Cloudy	Passing Clouds	Upstream
	P32 MV2 (U)	27/11/2019	Collected by supervisor	9	N/A	Partly Cloudy	Partly Cloudy	Upstream
	P33 MV2 (U)	27/11/2019	Collected by supervisor	9	N/A	Partly Cloudy	Partly Cloudy	Upstream
	P34 MV2 (S)	27/11/2019	Collected by supervisor	9	N/A	Partly Cloudy	Partly Cloudy	Stepping Stones
	P35 MV2 (S)	27/11/2019	Collected by supervisor	9	N/A	Partly Cloudy	Partly Cloudy	Stepping Stones
	P36 MV1 (D)	27/11/2019	Collected by supervisor	10	N/A	Partly Cloudy	Partly Cloudy	Downstream
	P37 MV1 (D)	27/11/2019	Collected by supervisor	10	N/A	Partly Cloudy	Partly Cloudy	Downstream
	P68 MV3 (S)	14/01/2020	Collected by supervisor	8	N/A	Clear Skies	Partly Cloudy	Stepping Stones
	P69 MV3 (S)	14/01/2020	Collected by supervisor	8	N/A	Clear Skies	Partly Cloudy	Stepping Stones
	P70 MV2 (D)	14/01/2020	Collected by supervisor	8	N/A	Clear Skies	Partly Cloudy	Downstream
	P71 MV2 (D)	14/01/2020	Collected by supervisor	8	N/A	Clear Skies	Partly Cloudy	Downstream
	P72 MV3 (U)	14/01/2020	Collected by supervisor	8	N/A	Clear Skies	Partly Cloudy	Upstream
	P73 MV3 (U)	14/01/2020	Collected by supervisor	8	N/A	Clear Skies	Partly Cloudy	Upstream

River Thames	P38 TV1	03/12/2019	07:41	2	N/A	Clear Skies	Partly Sunny	UT bus stop
	P39 TV1	03/12/2019	07:41	2	N/A	Clear Skies	Partly Sunny	UT bus stop
	P40 TV1	03/12/2019	07:41	2	N/A	Clear Skies	Partly Sunny	UT bus stop
					N/A			
River Wey	P89 WV1 (A)	27/02/2020	11:00	3	N/A	Partly Sunny	Light Rain and Cloudy	The Anchor
	P90 WV1 (UC)	27/02/2020	11:05	4	N/A	Partly Sunny	Light Rain and Cloudy	Canal (Up the Pub)
	P91 WV1 (UC)	27/02/2020	11:05	4	N/A	Partly Sunny	Light Rain and Cloudy	Canal (Up the Pub)
	P92 WV1 (B)	27/02/2020	11:20	4	N/A	Partly Sunny	Light Rain and Cloudy	Canal (Up the Pub)
	P93 WV1 (B)	27/02/2020	11:20	4	N/A	Partly Sunny	Light Rain and Cloudy	Canal (Up the Pub)
	P94 WV1 (B)	27/02/2020	11:20	4	N/A	Partly Sunny	Light Rain and Cloudy	Canal (Up the Pub)
	P95 WV1 (B)	27/02/2020	11:20	4	N/A	Partly Sunny	Light Rain and Cloudy	Canal (Up the Pub)
	P96 WV1 (S)	27/02/2020	11:37	4	N/A	Partly Sunny	Light Rain and Cloudy	Direct Sewage Outlet
	P97 WV1 (S)	27/02/2020	11:37	4	N/A	Partly Sunny	Light Rain and Cloudy	Direct Sewage Outlet
	P98 WV1 (S)	27/02/2020	11:37	4	N/A	Partly Sunny	Light Rain and Cloudy	Direct Sewage Outlet
	P99 WV1 (S)	27/02/2020	11:37	4	N/A	Partly Sunny	Light Rain and Cloudy	Direct Sewage Outlet

	P100 WV1 (S)	27/02/2020	11:37	4	N/A	Partly Sunny	Light Rain and Cloudy	Direct Sewage Outlet
	P101 WV1 (S)	27/02/2020	11:37	4	N/A	Partly Sunny	Light Rain and Cloudy	Direct Sewage Outlet
	P102 WV1 (S)	27/02/2020	11:37	4	N/A	Partly Sunny	Light Rain and Cloudy	Direct Sewage Outlet
	P103 WV1 (S)	27/02/2020	11:37	4	N/A	Partly Sunny	Light Rain and Cloudy	Direct Sewage Outlet
	P104 WV2 (UC)	12/08/2020	Collected by supervisor	15	N/A	Partly Sunny	Partly Sunny	Canal (Up the Pub)
	P105 WV2 (UC)	12/08/2020	Collected by supervisor	15	N/A	Partly Sunny	Partly Sunny	Canal (Up the Pub)
	P106 WV1 (DC)	12/08/2020	Collected by supervisor	15	N/A	Partly Sunny	Partly Sunny	Canal (Down the Pub)
	P107 WV2 (S)	12/08/2020	Collected by supervisor	15	N/A	Partly Sunny	Partly Sunny	Direct Sewage Outlet
	P108 WV2 (B)	12/08/2020	Collected by supervisor	15	N/A	Partly Sunny	Partly Sunny	Weybridge bridge
	P109 WV1 (G)	12/08/2020	Collected by supervisor	15	N/A	Partly Sunny	Partly Sunny	Golf Site new River Wey
	P110 WV1 (AB)	12/08/2020	Collected by supervisor	16	N/A	Partly Sunny	Partly Sunny	Abbey stream
	P111 WV1 (AB)	12/08/2020	Collected by supervisor	16	N/A	Partly Sunny	Partly Sunny	Abbey stream

Table 6:

Summary of phage isolated from four different Rivers using bovine faeces isolates.

Name of River	Phage Code	Bacteria infected
Hogsmill River	HV1 P1 (B)	*E. coli* Bin 312B
	HV2 P2 (B)	*E. coli* Bin 23E
	HV2 P3 (B)	*E. coli* Bin 24E
	HV2 P4 (B)	*E. coli* Bin 312B
	HV2 P5 (B)	*E. coli* DW 12B
	HV1 P6 (VW)	*E. coli* Bin 219C
	HV1 P7 (VW)	*E. coli* Bin 23E
	HV1 P8 (VW)	*E. coli* Bin 24E
	HV1 P9 (VW)	*E. coli* Bin 312B
	HV1 P10 (VW)	*E. coli* DW 12A
	HV1 P11 (VW)	*E. coli* DW 122B
	HV2 P24 (VW)	*E. coli* F2 16B
	HV2 P25 (VW)	*P. vulgaris* LV3 7E
	HV2 P26 (VW)	*P. vulgaris* LV3 22C
	HV1 P27 (U)	*E. coli* F2 8C
	HV1 P28 (U)	*E. coli* F2 16B
	HV1 P29 (P)	*E. coli* F2 8C
	HV1 P30 (D)	*E. coli* F2 8C
	HV1 P31 (D)	*E. coli* F2 16B
	HV1 P41 (R)	*P. vulgaris* LV3 11B
	HV1 P42 (R)	*P. vulgaris* LV3 19B
	HV1 P43 (K)	*P. vulgaris* LV3 11B
	HV1 P44 (K)	*P. mirabilis* DW V2 13C
	HV1 P45 (K)	*P. mirabilis* DW V2 4D
	HV3 P46 (VW)	*P. vulgaris* LV3 11B
	HV3 P47 (VW)	*P. vulgaris* LV3 19B
	HV3 P48 (B)	*P. vulgaris* LV3 11B
	HV3 P49 (B)	*P. vulgaris* LV3 19B
	HV3 P50 (B)	*P. vulgaris* LV3 23B

	HV4 P51 (VW)	*P. vulgaris* LV3 11B
	HV4 P52 (VW)	*P. vulgaris* LV3 23B
	HV4 P53 (B)	*E. coli* F2 10D
	HV4 P54 (B)	*E. coli* F2 K1A
	HV4 P55 (B)	*E.coli* LV3 10A
	HV4 P56 (B)	*Klebsiella* sp F2 3G
	HV4 P57 (B)	*P. aeruginosa* BV3 3E
	HV4 P58 (B)	*P. vulgaris* BV3 18D
	HV4 P59 (B)	*P. vulgaris* BV3 21B1
	HV4 P60 (B)	*P. vulgaris* BVT4 20C
	HV5 P61 (VW)	*E. coli* F2 4D
	HV5 P62 (VW)	*E. coli* F2 10D
	HV5 P63 (VW)	*E. coli* F2 K1A
	HV5 P64 (VW)	*E. coli* LV3 10A
	HV5 P65 (VW)	*E. coli* LV3 21B
	HV5 P66 (VW)	*Klebsiella* sp F2 3G
	HV5 P67 (VW)	*P. vulgaris* BV3 21B1
	HV2 P74 (K)	*E. coli* 25D
	HV2 P75 (K)	*E. coli* 24D2
	HV2 P76 (K)	*E. coli* 26B2
	HV6 P77 (VW)	*E. coli* 24D2
River Mole	MV1 P12 (S)	*E. coli* DWV1 22B2
	MV1 P13 (S)	*P. vulgaris* F2 4C
	MV1 P14 (S)	*P. vulgaris* LV3 10D
	MV1 P15 (S)	*P. aeruginosa* DWV1 22D
	MV2 P16 (S)	*E. coli* DWV1 22B2
	MV2 P17 (S)	*E. coli* DWV1 6A21
	MV2 P18 (S)	*P. vulgaris* F2 4C
	MV2 P19 (S)	*P. vulgaris* LV3 10D
	MV2 P20 (S)	*P. aeruginosa* DWV1 22D
	MV1 P21 (U)	*P. vulgaris* F2 4C
	MV1 P22 (U)	*P. vulgaris* LV3 10D

	MV1 P23 (U)	*P. aeruginosa* DWV1 22D
	MV2 P32 (U)	*E. coli* F2 8C
	MV2 P33 (U)	*E. coli* F2 16B
	MV2 P34 (S)	*E. coli* F2 8C
	MV2 P35 (S)	*E. coli* F2 16B
	MV1 P36 (D)	*E. coli* F2 8C
	MV1 P37 (D)	*E. coli* F2 16B
	MV3 P68 (S)	*E. coli* 20F
	MV3 P69 (S)	*E. coli* 21C
	MV2 P70 (D)	*E. coli* 21C
	MV2 P71 (D)	*E. coli* 24C1
	MV3 P72 (U)	*E. coli* 21C
	MV3 P73 (U)	*E. coli* 24C1
River Thames	TV1 P38	*P. vulgaris* LV3 11B
	TV1 P39	*P. vulgaris* LV3 19B
	TV1 P40	*P. vulgaris* LV3 23B
River Wey	WV1 P89 (A)	*E. coli* isolated at MV1 (S)
	WV1 P90 (UC)	*E. coli* isolated at HV1 (U)
	WV2 P104 (UC)	*E. coli* 9F
	WV2 P105 (UC)	*P. vulgaris* 7D2
	WV1 P106 (DC)	*E. coli* 9F
	WV2 P107 (S)	*E. coli* 9F
	WV2 P108 (B)	*E. coli* 9F
	WV1 P109 (G)	*E. coli* 9F
	WV1 P110 (AB)	*E. coli* 9F
	WV1 P111 (AB)	*P. vulgaris* 7D2

Table 7: Summary of phages isolated using bacteria from water samples		
Name of River	**Phage Code**	**Bacteria infected**
Hogsmill River	HV5 P78 (B)	*E. coli isolated at MV1 (S)*
	HV5 P79 (B)	*Klebsiella* sp isolated at MV1 (S)
	HV5 P80 (B)	*Proteus* sp isolated at MV1 (D)
	HV5 P81 (B)	*Proteus* sp isolated at MV1 (D)
	HV5 P82 (B)	*Proteus* sp isolated at MV1 (D)
	HV7 P83 (VW)	*Klebsiella* sp isolated at MV1 (S)
	HV7 P84 (VW)	*Pseudomonas* sp isolated at MV1 (D)
	HV7 P85 (VW)	*Pseudomonas* sp isolated at MV1 (D)
	HV7 P86 (VW)	*Pseudomonas* sp isolated at MV1 (D)
	HV7 P87 (VW)	*E. coli* isolated at Hogsmill HV2 (K)
	HV7 P88 (VW)	*Proteus* sp isolated at HV2 (K)
River Wey	WV1 P91 (UC)	*E. coli* isolated at MV1 (S)
	WV1 P92 (B)	*E. coli* isolated at MV1 (S)
	WV1 P93 (B)	*E. coli* isolated at HV7 (VW)
	WV1 P94 (B)	*E. coli* isolated at HV7 (VW)
	WV1 P95 (B)	*E. coli* isolated at HV7 (VW)
	WV1 P96 (S)	*E. coli* isolated at MV1 (S)
	WV1 P97 (S)	*Pseudomonas* sp isolated at MV2 (D)
	WV1 P98 (S)	*Pseudomonas* sp isolated at MV2 (D)
	WV1 P99 (S)	*Pseudomonas* sp isolated at MV1 (U)
	WV1 P100 (S)	*Pseudomonas* sp isolated at MV1 (U)
	WV1 P101 (S)	*E. coli* isolated at HV7 (VW)
	WV1 P102 (S)	*E. coli* isolated at HV7 (VW)
	WV1 P103 (S)	*E. coli* isolated at HV7 (VW)

Table 8: Determination of phage host range		
Date	**Phage Code**	**Bovine Faeces Bacteria Infected**
29/09/2020	HV1 P6 (VW)	*E. coli* BIN 23E
29/09/2020	HV2 P2 (B)	*E. coli* BIN 23E
29/09/2020	HV2 P3 (B)	*E. coli* BIN 23E
29/09/2020	HV2 P4 (B)	*E. coli* BIN 23E
29/09/2020	HV2 P5 (B)	*E. coli* BIN 23E
06/10/2020	HV5 P80 (B)	*E. coli* BIN 23E
06/10/2020	HV6 P87 (VW)	*E. coli* BIN 23E
06/10/2020	WV1 P89 (A)	*E. coli* BIN 23E
13/05/2021	HV2 P2 (B)	*E. coli* BV3 20C
13/05/2021	HV2 P3 (B)	*E. coli* BV3 20C
13/05/2021	HV1 P6 (VW)	*E. coli* BV3 20C
13/05/2021	HV2 P3 (B)	*E. coli* BV3 21B1
23/09/2020	HV1 P27 (U)	*E. coli* BV3 23B
23/09/2020	HV2 P25 (VW)	*E. coli* BV3 23B
23/09/2020	HV2 P26 (VW)	*E. coli* BV3 23B
23/09/2020	MV1 P14 (S)	*E. coli* BV3 23B
23/09/2020	MV2 P17 (S)	*E. coli* BV3 23B
29/09/2020	HV2 P2 (B)	*E. coli* BV3 23B
13/05/2021	HV1 P1 (B)	*E. coli* BV3 23B
13/05/2021	HV2 P3 (B)	*E. coli* BV3 23B
13/05/2021	HV2 P5 (B)	*E. coli* BV3 23B
13/05/2021	HV1 P6 (VW)	*E. coli* BV3 23B
13/05/2021	HV2 P3 (B)	*E. coli* BV3 24D
13/05/2021	HV2 P5 (B)	*E. coli* BV3 24D
13/05/2021	HV1 P6 (VW)	*E. coli* BV3 24D
13/05/2021	HV1 P6 (VW)	*E. coli* BV4 21C
15/10/2020	HV5 P77 (VW)	*E. coli* DWV1 21C
15/10/2020	MV2 P71 (D)	*E. coli* DWV1 21C
15/10/2020	MV3 P68 (S)	*E. coli* DWV1 21C
15/10/2020	HV2 P74 (K)	*E. coli* DWV1 24D2
15/10/2020	HV2 P76 (K)	*E. coli* DWV1 24D2
17/09/2020	HV2 P25 (VW)	*E. coli* DWV1 25D
17/09/2020	HV4 P56 (B)	*E. coli* DWV1 25D
17/09/2020	MV2 P17 (S)	*E. coli* DWV1 25D
22/09/2020	HV1 P27 (U)	*E. coli* DWV1 25D
22/09/2020	HV2 P25 (VW)	*E. coli* DWV1 25D
22/09/2020	MV1 P14 (S)	*E. coli* DWV1 25D
22/09/2020	MV2 P17 (S)	*E. coli* DWV1 25D
27/10/2020	WV2 P106 (DC)	*E. coli* DWV2 11B1
27/10/2020	WV2 P106 (DC)	*E. coli* DWV2 11E
27/10/2020	WV2 P106 (DC)	*E. coli* DWV2 12F
27/10/2020	WV2 P106 (DC)	*E. coli* DWV2 14D
15/10/2020	HV5 P77 (VW)	*E. coli* DWV2 20F
15/10/2020	MV3 P69 (S)	*E. coli* DWV2 20F

27/10/2020	WV1 P103 (S)	*E. coli* DWV2 9F
27/10/2020	WV2 P104 (UC)	*E. coli* DWV2 9F
27/10/2020	WV2 P105 (UC)	*E. coli* DWV2 9F
27/10/2020	WV2 P106 (DC)	*E. coli* DWV2 9F
17/09/2020	MV1 P14 (S)	*E. coli* F216B
22/09/2020	HV2 P25 (VW)	*E. coli* F2 16B
22/09/2020	HV2 P26 (VW)	*E. coli* F2 16B
23/09/2020	HV1 P27 (U)	*E. coli* F2 1E
23/09/2020	HV2 P25 (VW)	*E. coli* F2 1E
23/09/2020	HV2 P26 (VW)	*E. coli* F2 1E
30/09/2020	HV2 P3 (B)	*E. coli* F2 4D
06/10/2020	HV5 P80 (B)	*E. coli* F2 4D
06/10/2020	HV6 P87 (VW)	*E. coli* F2 4D
06/10/2020	WV1 P89 (A)	*E. coli* F2 4D
23/09/2020	HV2 P26 (VW)	*E. coli* LV3 15C
23/09/2020	MV1 P14 (S)	*E. coli* LV3 15C
23/09/2020	MV2 P17 (S)	*E. coli* LV3 15C
29/09/2020	HV1 P1 (B)	*E. coli* LV3 15C
29/09/2020	HV2 P3 (B)	*E. coli* LV3 15C
29/09/2020	HV2 P4 (B)	*E. coli* LV3 15C
23/09/2020	HV2 P26 (VW)	*E. coli* LV3 21B
23/09/2020	MV1 P14 (S)	*E. coli* LV3 21B
23/09/2020	MV2 P17 (S)	*E. coli* LV3 21B
29/09/2020	HV2 P2 (B)	*E. coli* LV3 21B
06/10/2020	HV5 P80 (B)	*E. coli* LV3 21B
06/10/2020	WV1 P89 (A)	*E. coli* LV3 21B
30/09/2020	HV2 P5 (B)	*E. coli* LV3 7E
23/09/2020	HV1 P27 (U)	*P. vulgaris* BV3 10D
23/09/2020	HV2 P25 (VW)	*P. vulgaris* BV3 10D
23/09/2020	MV1 P14 (S)	*P. vulgaris* BV3 10D
23/09/2020	MV2 P17 (S)	*P. vulgaris* BV3 10D
17/09/2020	HV2 P25 (VW)	*P. vulgaris* F24C
22/09/2020	HV1 P27 (U)	*P. vulgaris* F24C
22/09/2020	HV2 P26 (VW)	*P. vulgaris* F24C
30/09/2020	HV1 P6 (VW)	*P. vulgaris* LV3 10D
30/09/2020	HV2 P2 (B)	*P. vulgaris* LV3 10D
30/09/2020	HV2 P3 (B)	*P. vulgaris* LV3 10D
30/09/2020	HV2 P4 (B)	*P. vulgaris* LV3 10D
30/09/2020	HV2 P5 (B)	*P. vulgaris* LV3 10D

Table 9: Determination of phage host range against human isolates		
Date	**Phage Code**	**Human Isolates**
12/11/2020	MV1 P15 (S)	*E. coli* 2
26/11/2020	HV1 P43 (K)	*E. coli* 2
26/11/2020	HV1 P47 (VW2)	*E. coli* 2
12/11/2020	MV2 P16 (S)	*E. coli* 2
19/11/2020	MV2 P19 (S)	*E. coli* 2
19/11/2020	MV2 P20 (S)	*E. coli* 2
26/11/2020	HV1 P43 (K)	*E. coli* 2P
26/11/2020	HV1 P46 (VW2)	*E. coli* 2P
26/11/2020	HV1 P47 (VW2)	*E. coli* 2P
19/11/2020	HV1 P6 (VW)	*E. coli* 2P
04/12/2020	HV4 P53 (B)	*E. coli* 2P
04/12/2020	HV4 P61 (VW)	*E. coli* 2P
04/12/2020	HV4 P62 (VW)	*E. coli* 2P
04/12/2020	HV4 P64 (VW)	*E. coli* 2P
26/11/2020	MV2 P32(U)	*E. coli* 2P
26/11/2020	MV2 P34 (S)	*E. coli* 2P
26/11/2020	TV1 P39	*E. coli* 2P
26/11/2020	HV1 P47 (VW2)	*E. coli* 3P
12/11/2020	HV1 P6 (VW)	*E. coli* 3P
26/11/2020	HV1 P43 (K)	*E. coli* 3P
26/11/2020	HV3 P51 (VB)	*E. coli* 4P
26/11/2020	MV2 P32(U)	*E. coli* 4P
26/11/2020	HV1 P28 (U)	*E. coli* 5P
26/11/2020	HV1 P31 (D)	*E. coli* 5P
19/11/2020	HV1 P6 (VW)	*E. coli* 5P
19/11/2020	HV2 P4 (B)	*E. coli* 5P
19/11/2020	HV2 P5 (B)	*E. coli* 5P
19/11/2020	MV2 P19 (S)	*E. coli* 5P
19/11/2020	MV2 P20 (S)	*E. coli* 5P
12/11/2020	HV1 P1 (B)	*E. coli* 6
26/11/2020	HV1 P43 (K)	*E. coli* 6
12/11/2020	HV1 P6 (VW)	*E. coli* 6
12/11/2020	HV2 P4 (B)	*E. coli* 6
12/11/2020	HV2 P5 (B)	*E. coli* 6
26/11/2020	HV3 P49 (B)	*E. coli* 6
05/11/2020	HV1 P6 (VW)	*E. coli* 6P
05/11/2020	HV2 P3 (B)	*E. coli* 6P
05/11/2020	HV2 P4 (B)	*E. coli* 6P
26/11/2020	HV3 P49 (B)	*E. coli* 6P
19/11/2020	MV1 P23 (U)	*E. coli* 6P
19/11/2020	MV2 P16 (S)	*E. coli* 6P
19/11/2020	MV2 P18 (S)	*E. coli* 6P
26/11/2020	HV1 P43 (K)	*E. coli* 7P
19/11/2020	MV2 P16 (S)	*E. coli* 7P
19/11/2020	MV2 P18 (S)	*E. coli* 7P

12/11/2020 04/12/2020	HV1 P6 (VW) HV4 P53 (B)	*E. coli* 10P *E. coli* 10P
12/11/2020 12/11/2020	HV1 P7 (VW) MV1 P15 (S)	*E. coli* 11P *E. coli* 11P

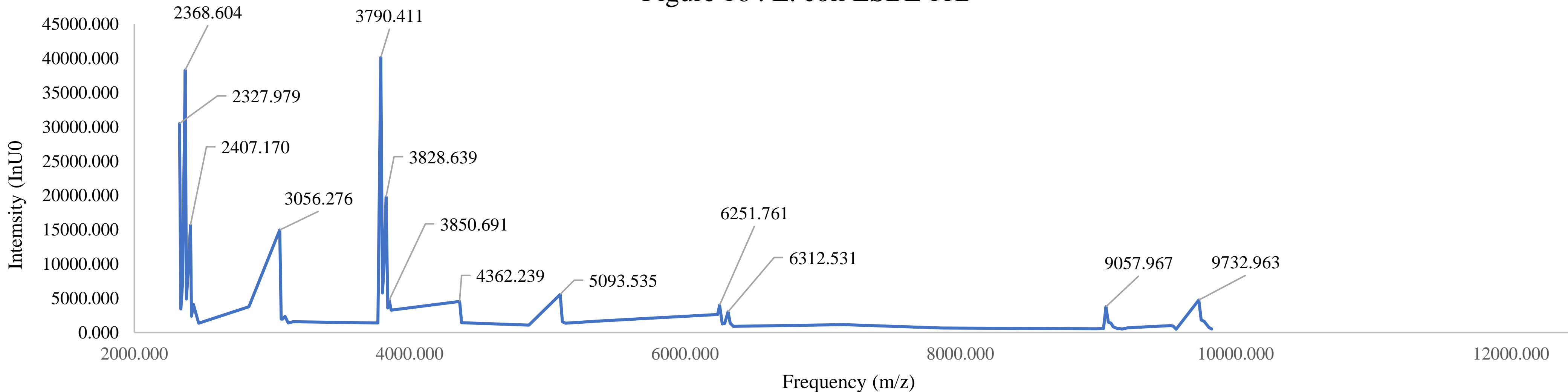
Table 10:
Determination of phage activity when diluted to 10^{-8} pfu/ml

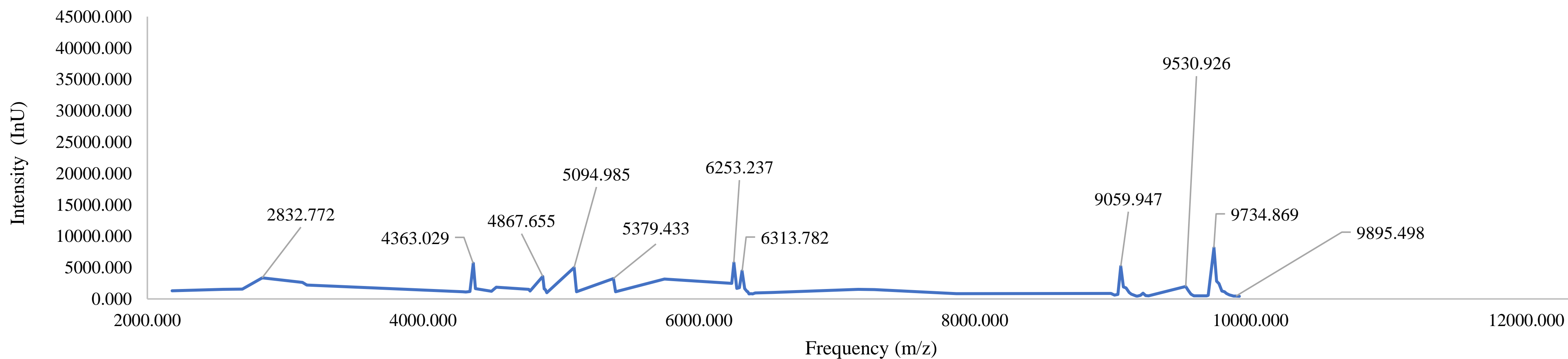
Date	**Phage Code**	**Bovine Faeces Isolates**	**Killing Titre Concentration pfu/ml**
03/12/2020	HV1 P27 (U)	*E. coli* F2 8C	10^{-2}
03/12/2020	HV1 P29 (P)	*E. coli* F2 8C	10^{-3}
03/12/2020	HV1 P31 (D)	*E. coli* F2 16B	10^{-2}
03/12/2020	MV2 P32 (U)	*E. coli* F2 8C	10^{-3}
03/12/2020	MV2 P33 (U)	*E. coli* F2 16B	10^{-1}
09/12/2020	MV3 P69 (S)	*E. coli* 21C	10^{-3}
09/12/2020	MV3 P72 (U)	*E. coli* 21C	10^{-4}
16/12/2020	MV1 P15 (S)	*P. aeruginosa* DWV1 22D	10^{-3}
16/12/2020	MV2 P20 (S)	*P. aeruginosa* DWV1 22D	10^{-1}
16/12/2020	MV1 P21 (U)	*P. vulgaris* F2 4C	10^{-3}
16/12/2020	MV1 P23 (U)	*P. aeruginosa* DWV1 22D	10^{-3}
16/12/2020	MV1 P23 (U)	*P. vulgaris* F2 4C	10^{-2}
24/03/2021	WV2 P104 (UC)	*E. coli* 9F	10^{-5}
24/03/2021	WV1 P106 (DC)	*E. coli* 9F	10^{-5}
24/03/2021	WV2 P107 (S)	*E. coli* 9F	10^{-5}
24/03/2021	WV2 P108 (B)	*E. coli* 9F	10^{-5}
24/03/2021	WV1 P109 (G)	*E. coli* 9F	10^{-8} – active at a dilution $>10^{-8}$
24/03/2021	WV1 P110 (AB)	*E. coli* 9F	10^{-8} – active at a dilution $>10^{-8}$
31/03/2021	HV1 P27 (U)	*E. coli* F2 8C	10^{-4}
31/03/2021	HV1 P28 (U)	*E. coli* F2 16B	10^{-5}
31/03/2021	HV1 P30 (D)	*E. coli* F2 8C	10^{-3}
31/03/2021	MV2 P33 (U)	*E. coli* F2 16B	10^{-4}
31/03/2021	MV1 P36 (D)	*E. coli* F2 8C	10^{-5}
31/03/2021	MV1 P37 (D)	*E. coli* F2 16B	10^{-3}
07/04/2021	HV4 P53 (B)	*E. coli* F2 10D	10^{-3}
07/04/2021	HV5 P62 (VW)	*E. coli* F2 10D	10^{-2}
07/04/2021	MV2 P70 (D)	*E. coli* DWV1 21C	10^{-6}
07/04/2021	MV3 P72 (U)	*E. coli* DWV1 21C	10^{-5}
14/04/2021	MV2 P17 (S)	*E. coli* DWV1 6A21	10^{-4}
16/04/2021	HV2 P5 (B)	*E. coli* DWV1 2B	10^{-6}

21/04/2021	HV4 P55 (B)	*E. coli* LV3 10A	10^{-6}
06/05/2021 06/05/2021 06/05/2021 06/05/2021	MV2 P71 (D) HV2 P74 (K) HV2 P76 (K) HV6 P77 (VW)	*E. coli* DWV1 21C *E. coli* DWV2 20F *E. coli* DWV1 24d2 *E. coli* DWV1 24d2	10^{-6} 10^{-8} – active at a dilution >10^{-8} 10^{-4} 10^{-1}

Table 11: Determination of phage activity when diluted to 10^{-8} pfu/ml			
Date	**Phage Code**	**Human ESBL Isolates**	**Killing Titre Concentration pfu/ml**
09/12/2020 09/12/2020 09/12/2020 09/12/2020 09/12/2020 09/12/2020 09/12/2020 09/12/2020	HV2 P3 (B) HV2 P4 (B) HV1 P6 (VW) HV1 P43 (K) HV1 P43 (K) HV3 P47 (VW) HV4 P53 (B) HV5 P61 (VW)	*E. coli* 16P *E. coli* 16P *E. coli* 16P *E. coli* 2P *E. coli* 6 *E. coli* 2P *E. coli* 2P *E. coli* 2P	10^{-3} 10^{-2} 10^{-1} 10^{-3} 10^{-1} 10^{-4} 10^{-1} 10^{-3}
16/04/2021	HV2 P4 (B)	*E. coli* 6	10^{-8} – active at a dilution >10^{-8}
21/04/2021	HV2 P3 (B)	*E. coli* 6	10^{-5}
28/04/2021	MV1 P23 (U)	*E. coli* 6P	10^{-3}
29/04/2021 29/04/2021 29/04/2021	HV4 P58 (B) HV5 P62 (VW) HV5 P64 (VW)	*E. coli* 10P *E. coli* 2P *E. coli* 2P	10^{-4} 10^{-3} 10^{-5}

APPENDIX C : MALDI – TOF GRAPHS AND TABLES





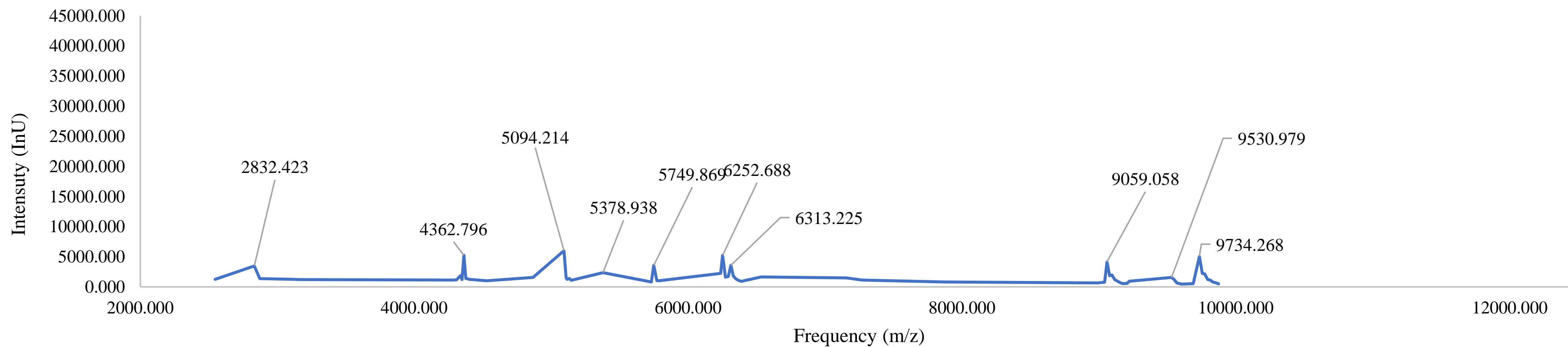


Figure 18: E. coli ESBL 6P
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensuty (InU)
2832.423
4362.796
5094.214
5378.938
5749.869
6252.688
6313.225
9059.058
9530.979
9734.268
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

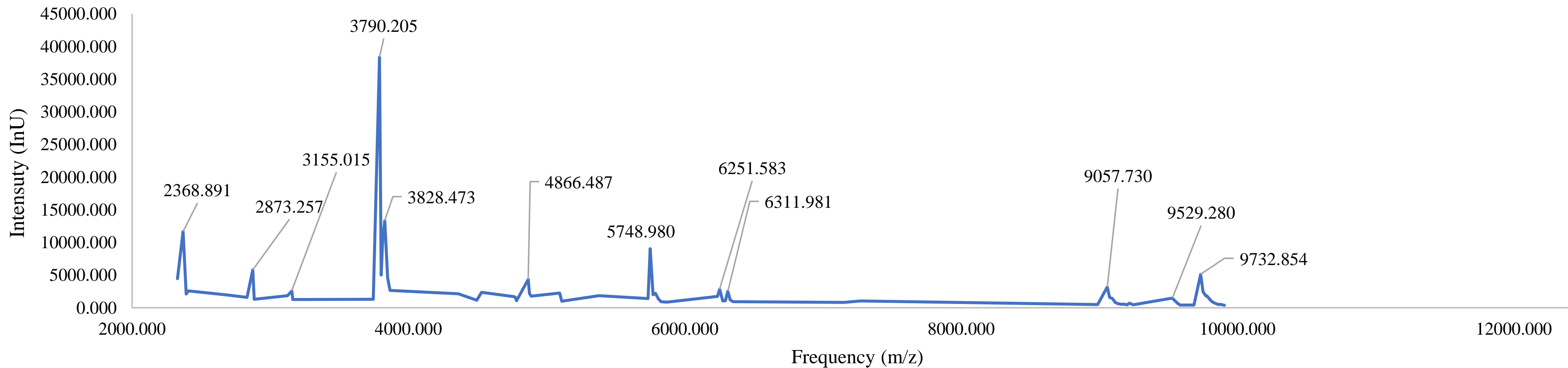


Figure 19: E. coli ESBL 6
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensuty (InU)
3790.205
3155.015
2368.891
2873.257
3828.473
4866.487
5748.980
6251.583
6311.981
9057.730
9529.280
9732.854
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

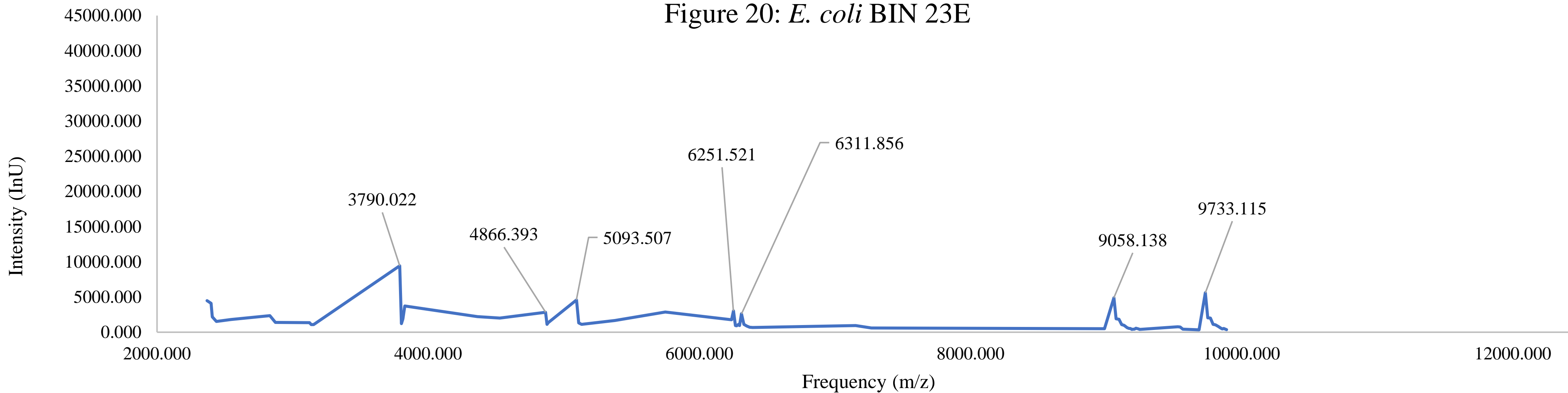


Figure 20: E. coli BIN 23E
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
3790.022
4866.393
5093.507
6251.521
6311.856
9058.138
9733.115

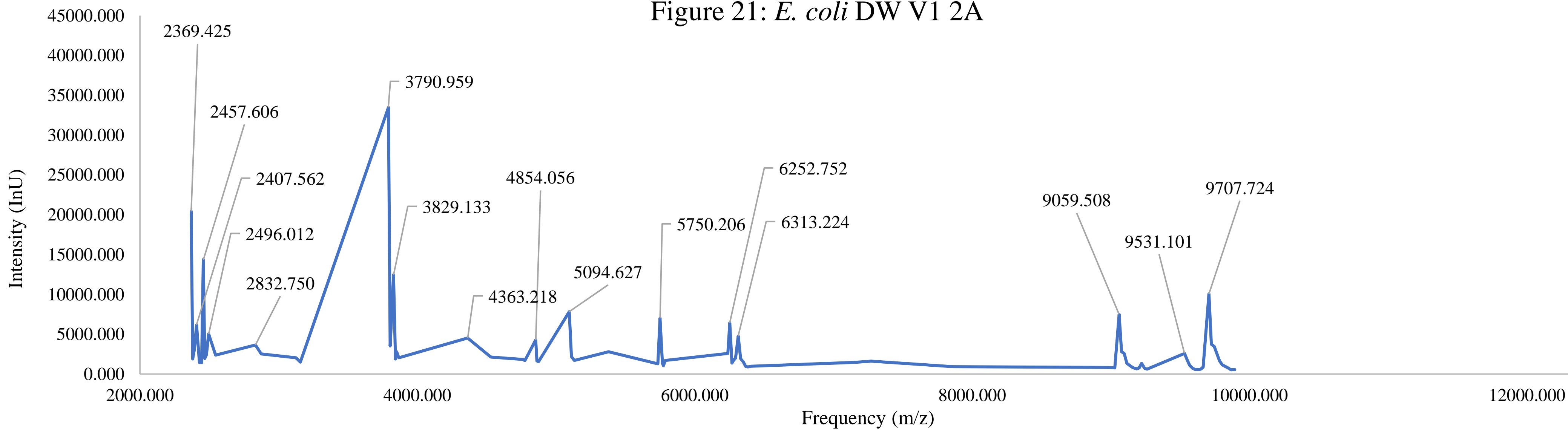


Figure 21: E. coli DW V1 2A
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2369.425
2457.606
2407.562
2496.012
2832.750
3790.959
3829.133
4363.218
4854.056
5094.627
5750.206
6252.752
6313.224
9059.508
9531.101
9707.724

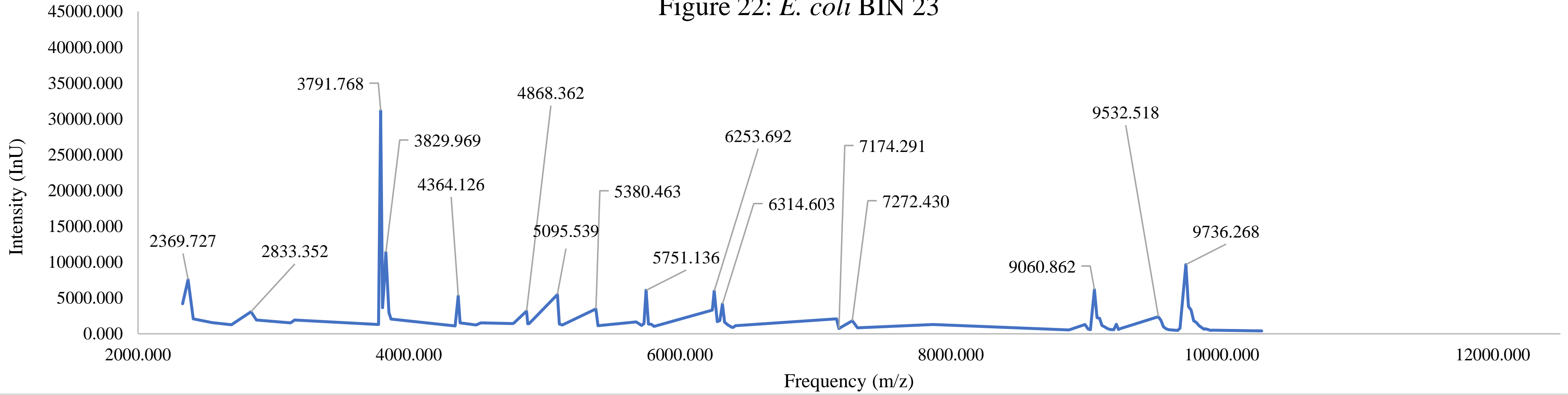


Figure 22: *E. coli* BIN 23

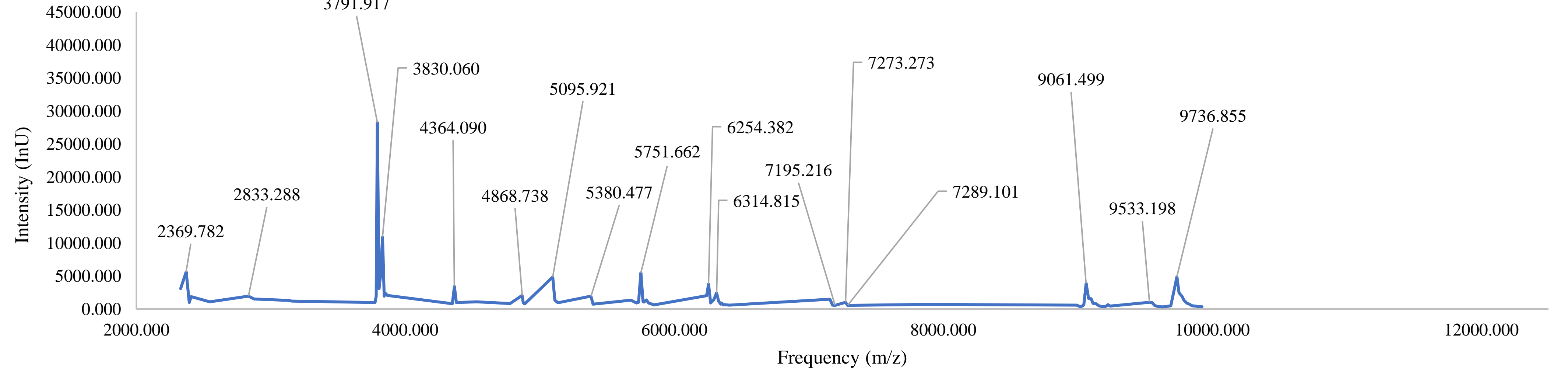


Figure 23: *E. coli* F2 10D

Figure 24: *E. coli* F2 4D

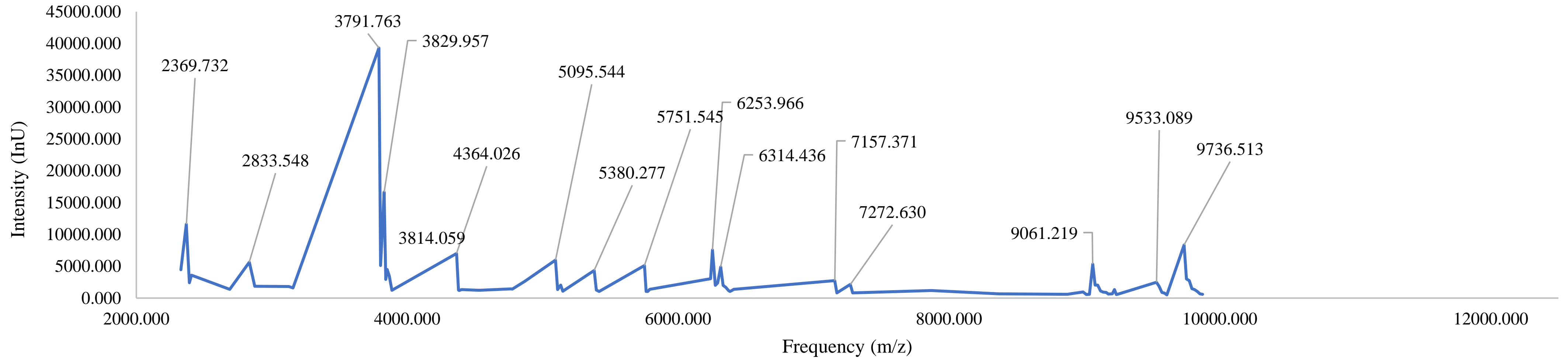
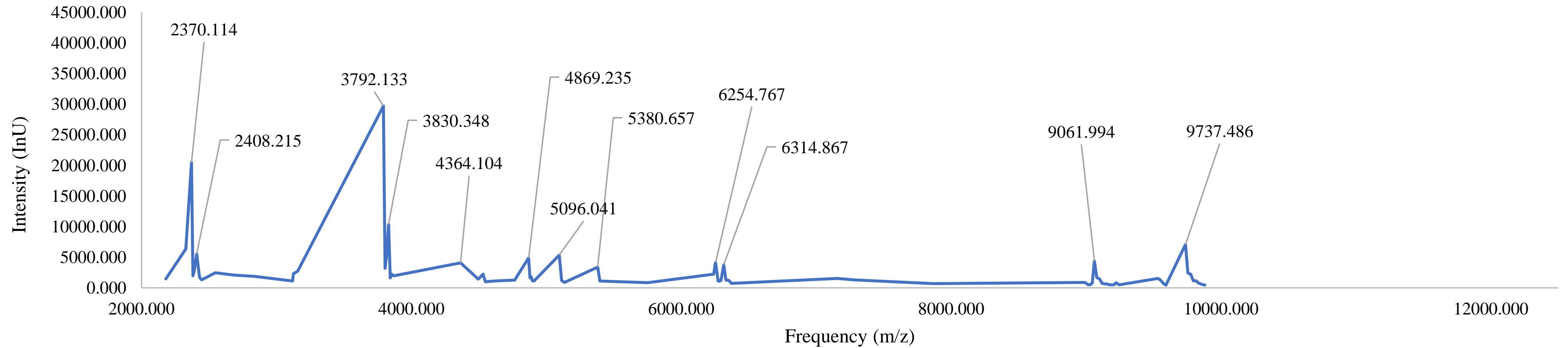


Figure 25: *E. coli* F2 K1A



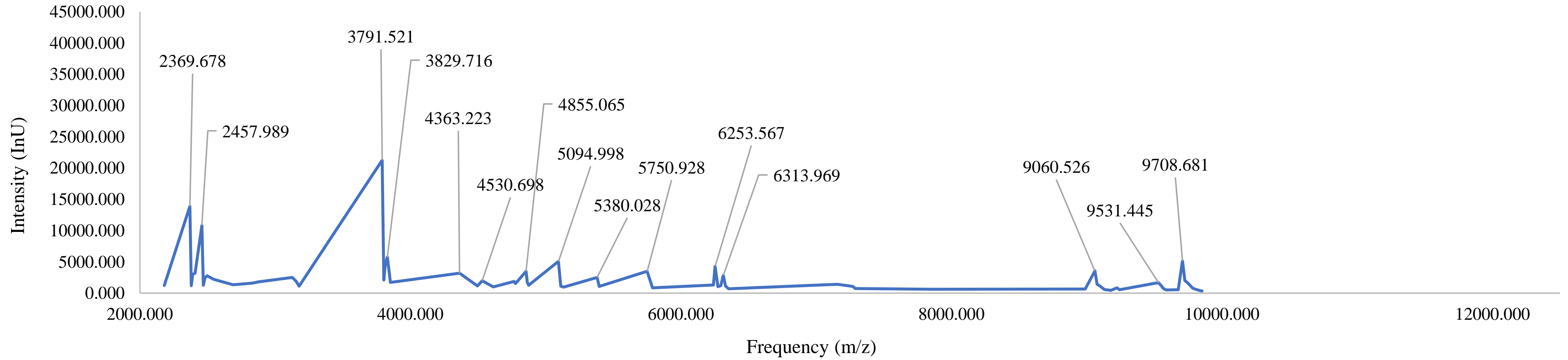


Figure 26: E. coli DW V1 22B2
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2369.678
2457.989
3791.521
3829.716
4363.223
4530.698
4855.065
5094.998
5380.028
5750.928
6253.567
6313.969
9060.526
9531.445
9708.681

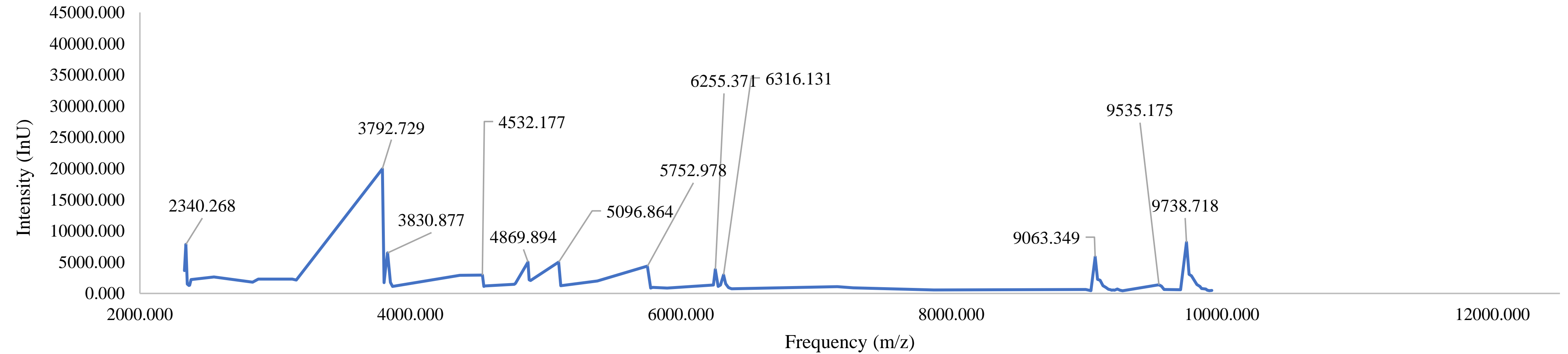


Figure 27: E. coli LV3 21B
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2340.268
3792.729
3830.877
4532.177
4869.894
5096.864
5752.978
6255.371
6316.131
9063.349
9535.175
9738.718

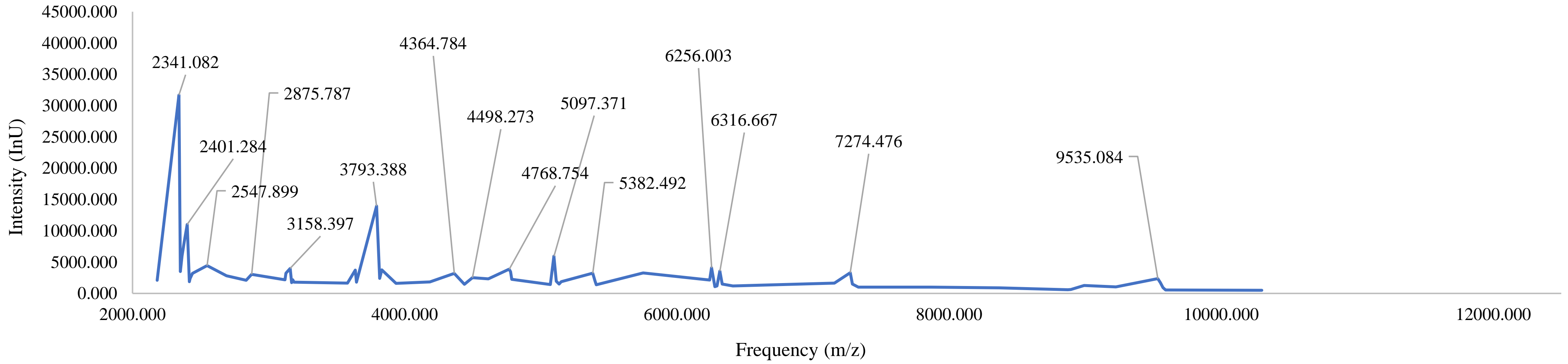


Figure 28: *E. coli* ESBL 5
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensity (InU)
2341.082
2401.284
2547.899
2875.787
3158.397
3793.388
4364.784
4498.273
4768.754
5097.371
5382.492
6256.003
6316.667
7274.476
9535.084
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

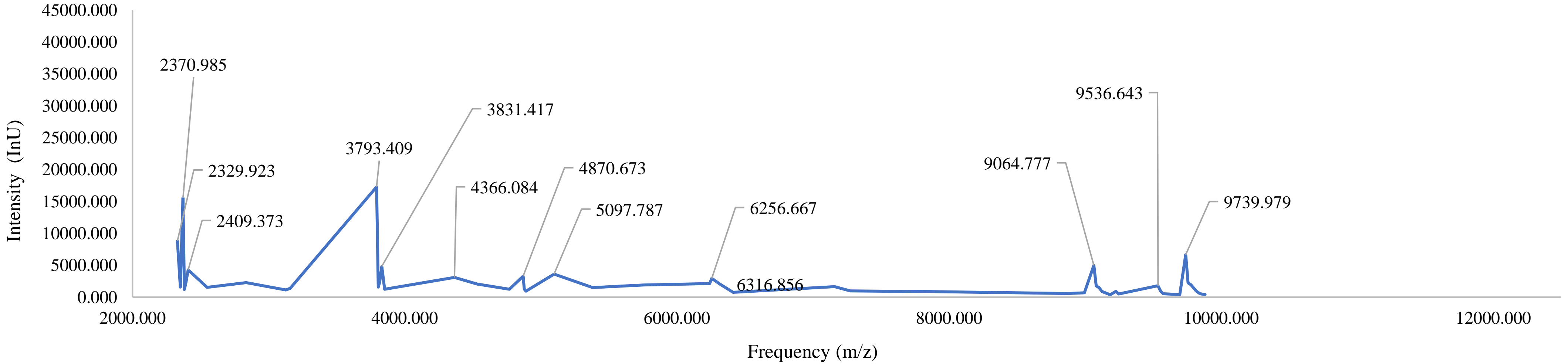


Figure 29: *E. coli* ESBL 7P
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensity (InU)
2370.985
2329.923
2409.373
3793.409
3831.417
4366.084
4870.673
5097.787
6256.667
6316.856
9064.777
9536.643
9739.979
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

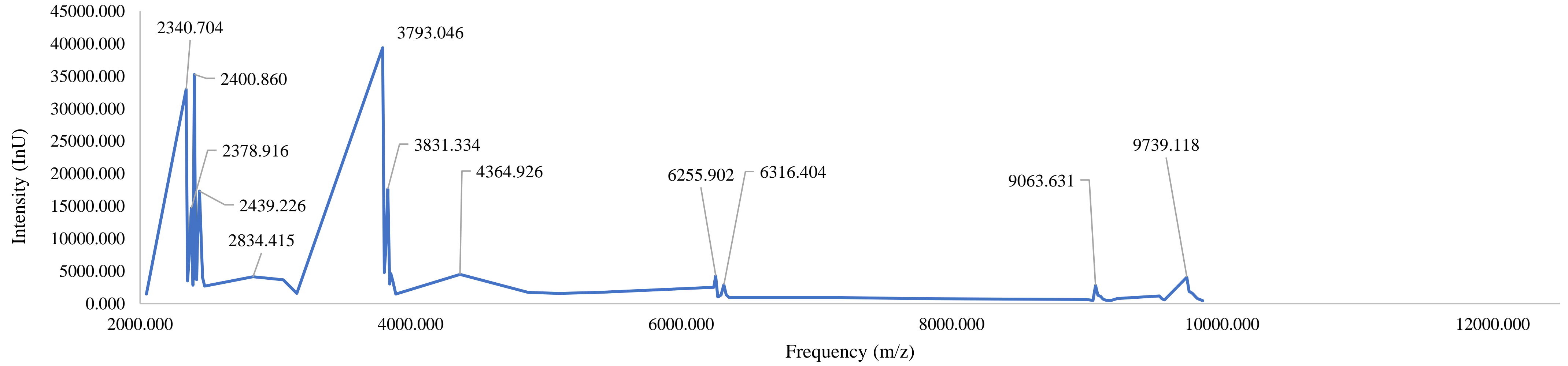


Figure 30: E. coli ESBL 9
Intensity (InU)
Frequency (m/z)
2340.704
2400.860
2378.916
2439.226
2834.415
3793.046
3831.334
4364.926
6255.902
6316.404
9063.631
9739.118

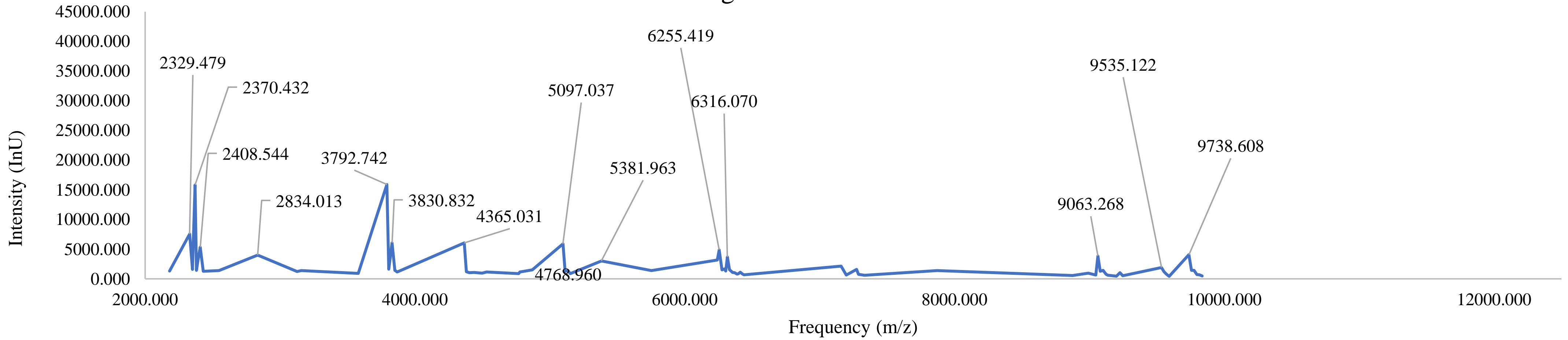


Figure 31: E.coli ESBL 9
Intensity (InU)
Frequency (m/z)
2329.479
2370.432
2408.544
2834.013
3792.742
3830.832
4365.031
4768.960
5097.037
5381.963
6255.419
6316.070
9063.268
9535.122
9738.608

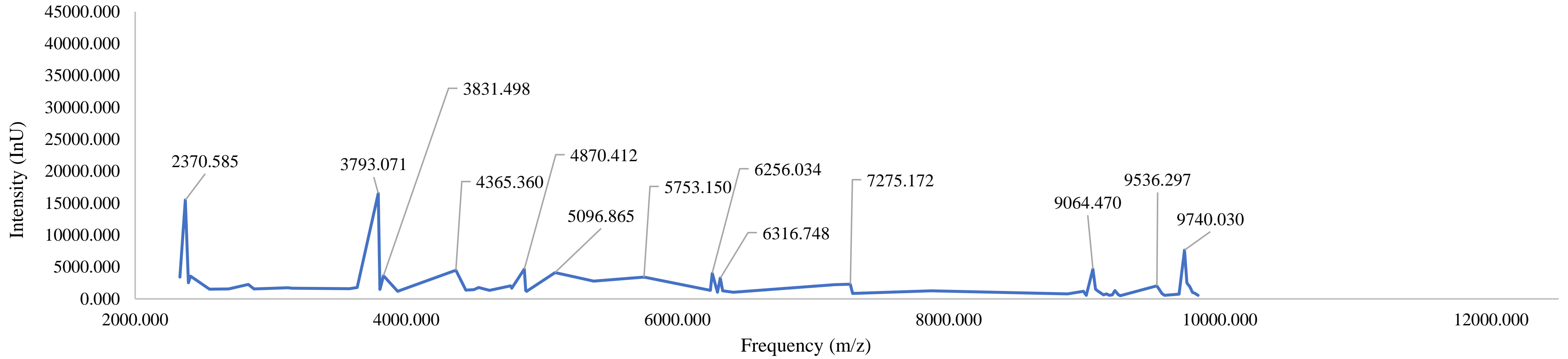


Figure 31: E. coli ESBL 2
Intensity (InU)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2370.585
3793.071
3831.498
4365.360
4870.412
5096.865
5753.150
6256.034
6316.748
7275.172
9064.470
9536.297
9740.030
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

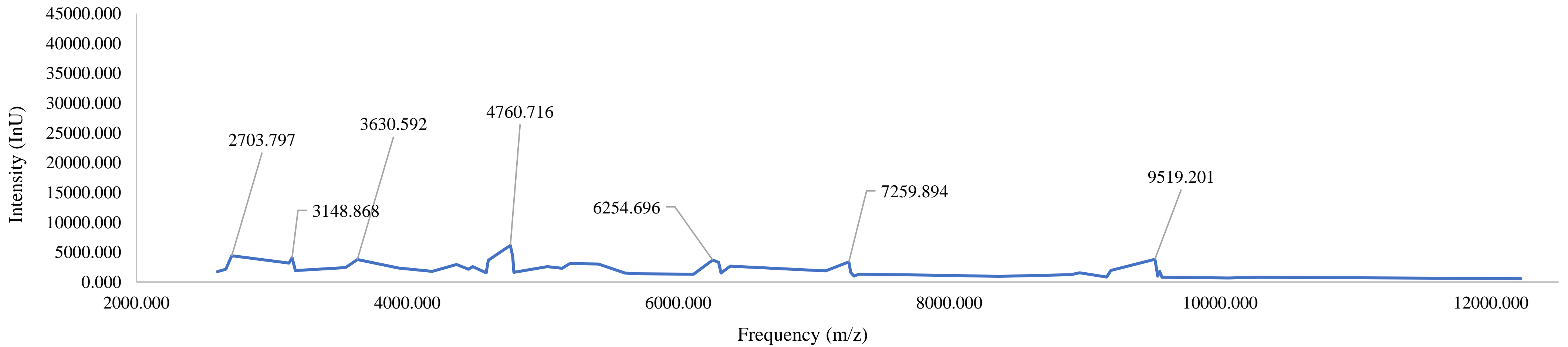


Figure 32: E. coli ESBL 1P
Intensity (InU)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2703.797
3148.868
3630.592
4760.716
6254.696
7259.894
9519.201
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

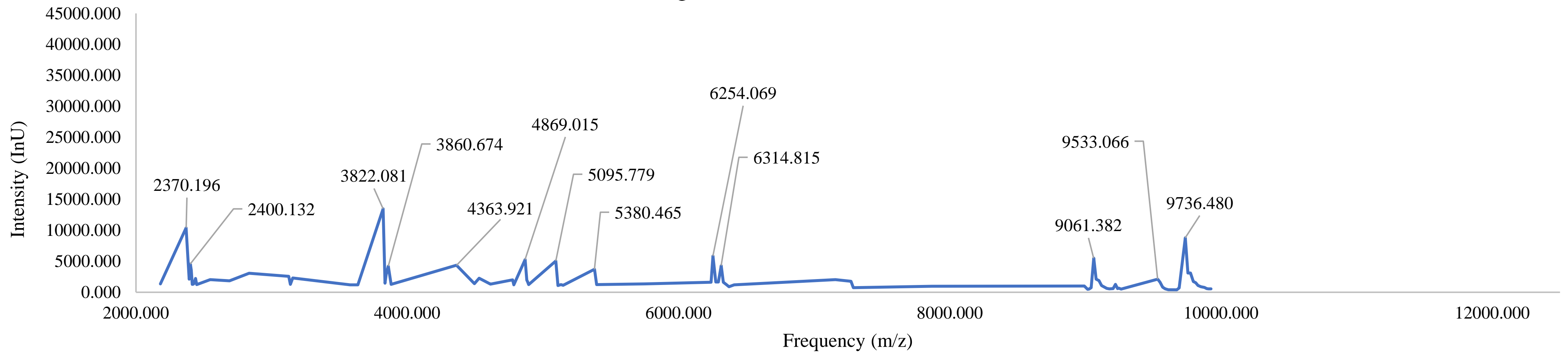


Figure 33: E. coli ESBL 5P
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensity (InU)
2370.196
2400.132
3822.081
3860.674
4363.921
4869.015
5095.779
5380.465
6254.069
6314.815
9533.066
9061.382
9736.480
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

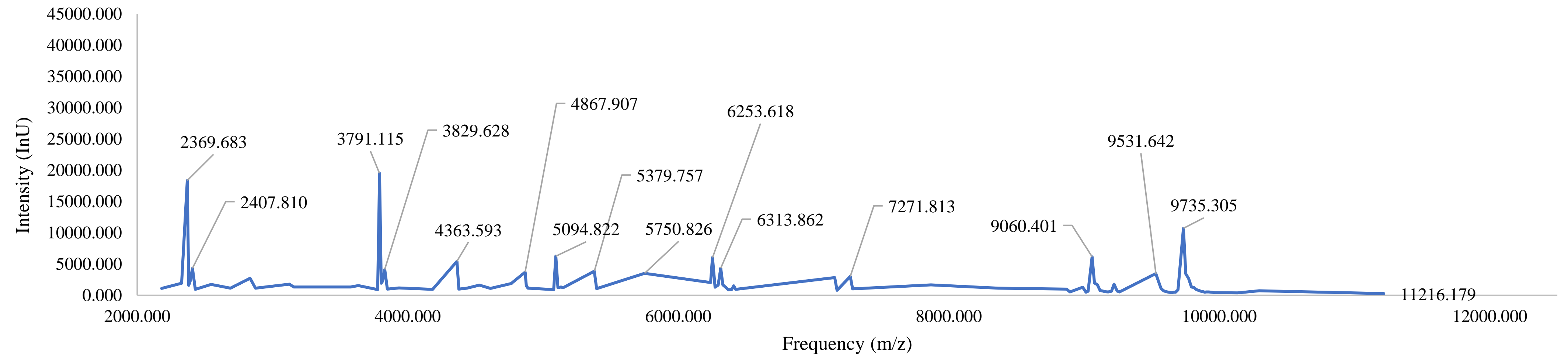


Figure 34: E. coli ESBL 4P
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensity (InU)
2369.683
2407.810
3791.115
3829.628
4363.593
4867.907
5094.822
5379.757
5750.826
6253.618
6313.862
7271.813
9060.401
9531.642
9735.305
11216.179
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

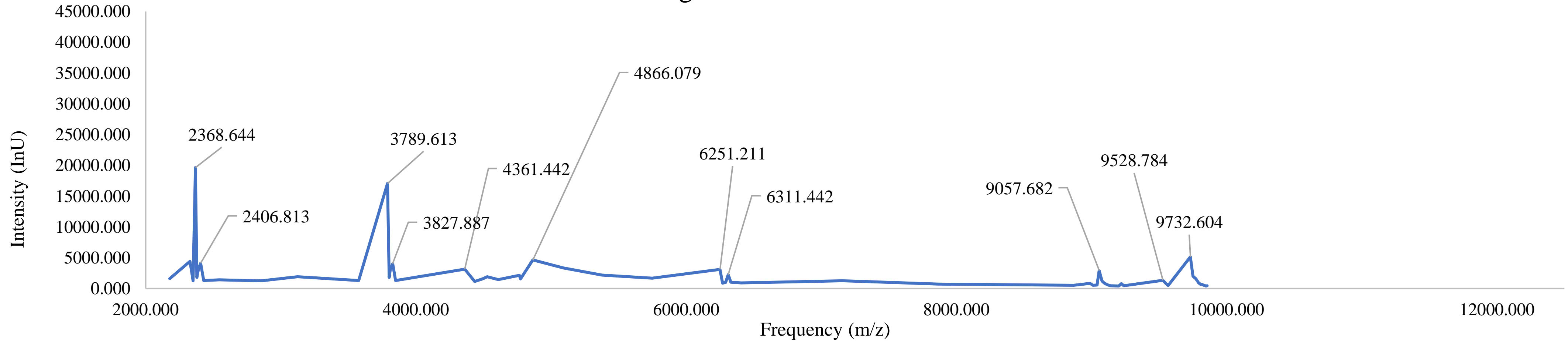


Figure 35: E. coli ESBL 7
Intensity (InU)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2368.644
2406.813
3789.613
3827.887
4361.442
4866.079
6251.211
6311.442
9057.682
9528.784
9732.604
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

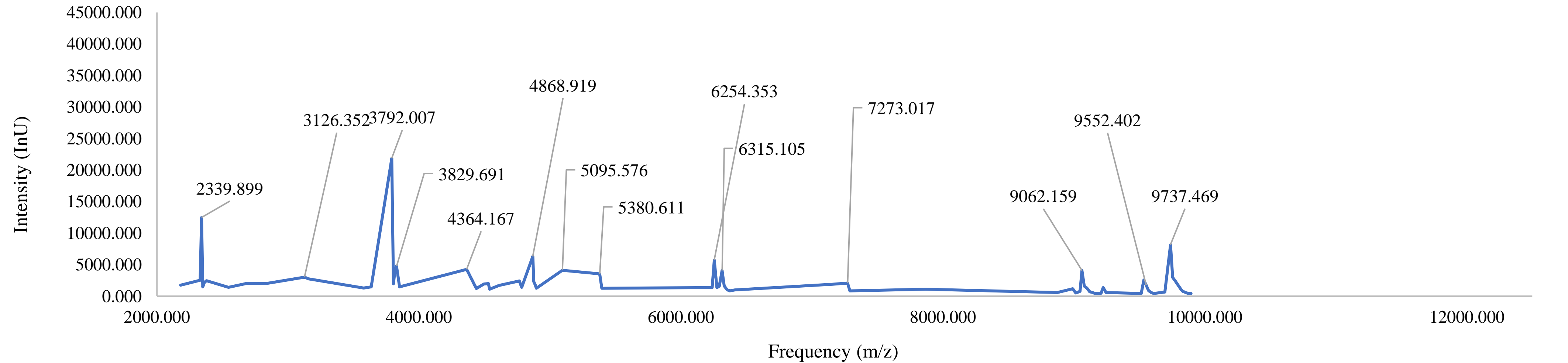


Figure 36: E. coli DW V1 26B2
Intensity (InU)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2339.899
3126.352
3792.007
3829.691
4364.167
4868.919
5095.576
5380.611
6254.353
6315.105
7273.017
9062.159
9552.402
9737.469
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

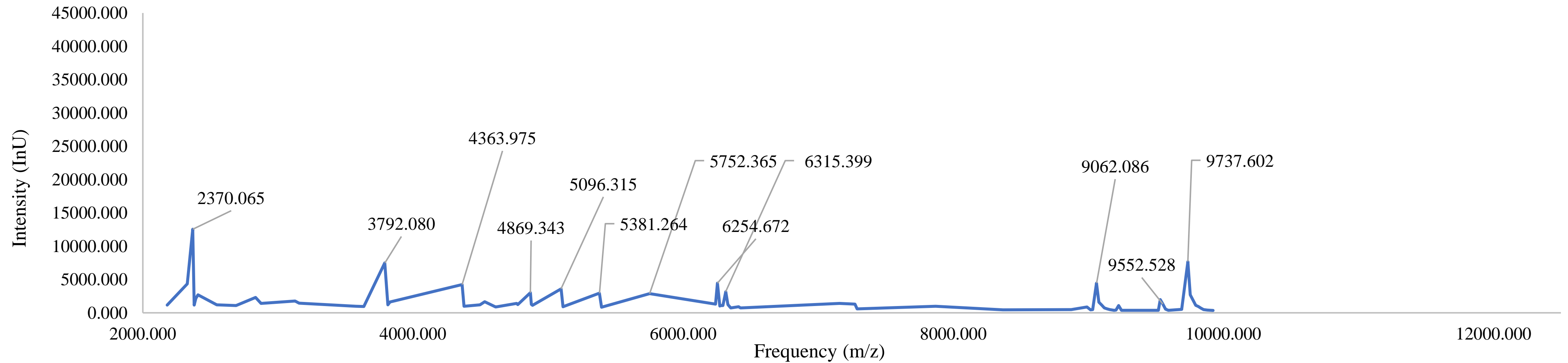


Figure 37: E. coli DW V1 25D
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2370.065
3792.080
4363.975
4869.343
5096.315
5381.264
5752.365
6254.672
6315.399
9062.086
9552.528
9737.602

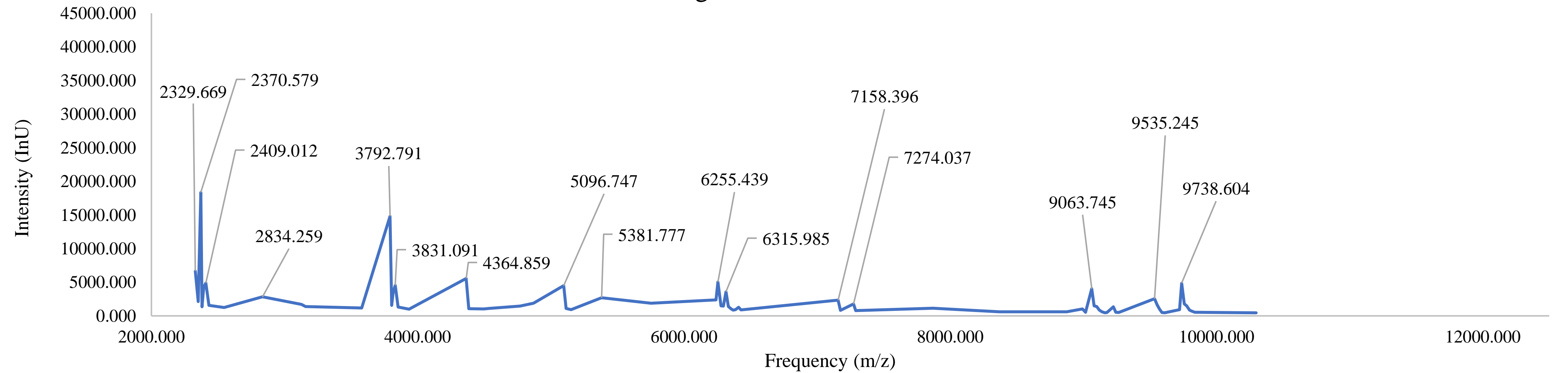


Figure 38: E. coli F2 16B
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2329.669
2370.579
2409.012
2834.259
3792.791
3831.091
4364.859
5096.747
5381.777
6255.439
6315.985
7158.396
7274.037
9063.745
9535.245
9738.604

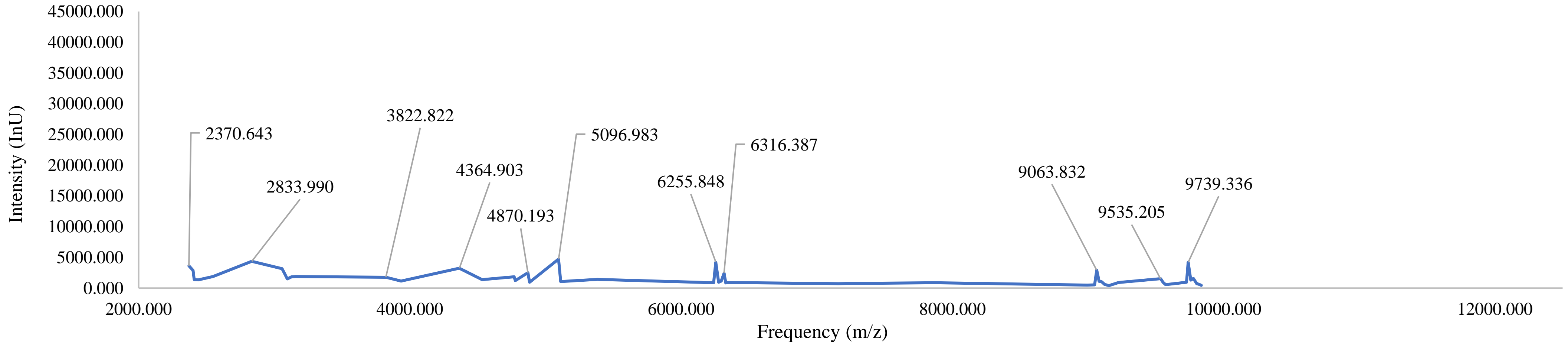


Figure 39: E. coli F2 8C
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensity (InU)
2370.643
2833.990
3822.822
4364.903
4870.193
5096.983
6255.848
6316.387
9063.832
9535.205
9739.336
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

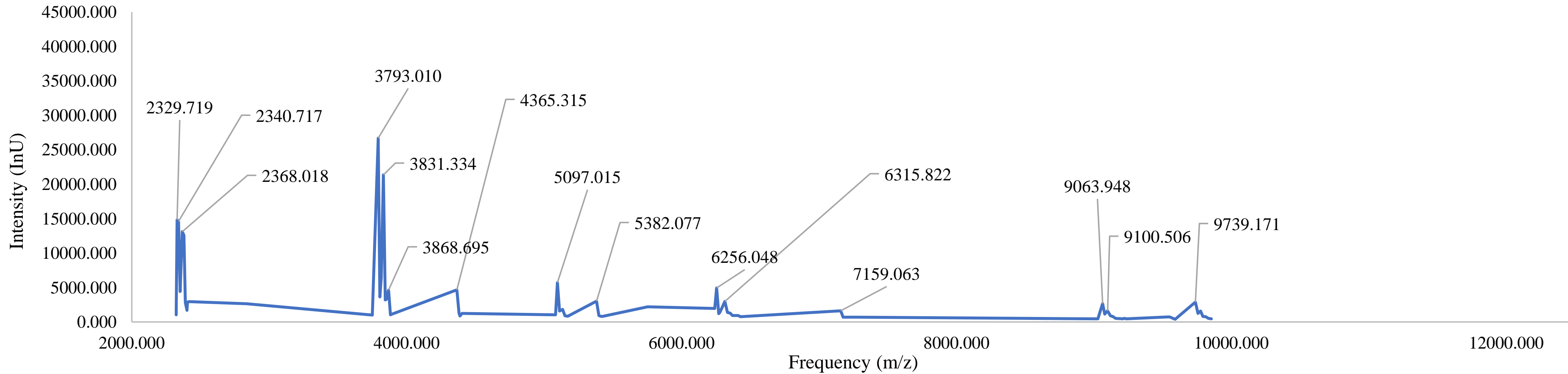


Figure 40: E. coli DW V1 2B
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensity (InU)
2329.719
2340.717
2368.018
3793.010
3831.334
3868.695
4365.315
5097.015
5382.077
6256.048
6315.822
7159.063
9063.948
9100.506
9739.171
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

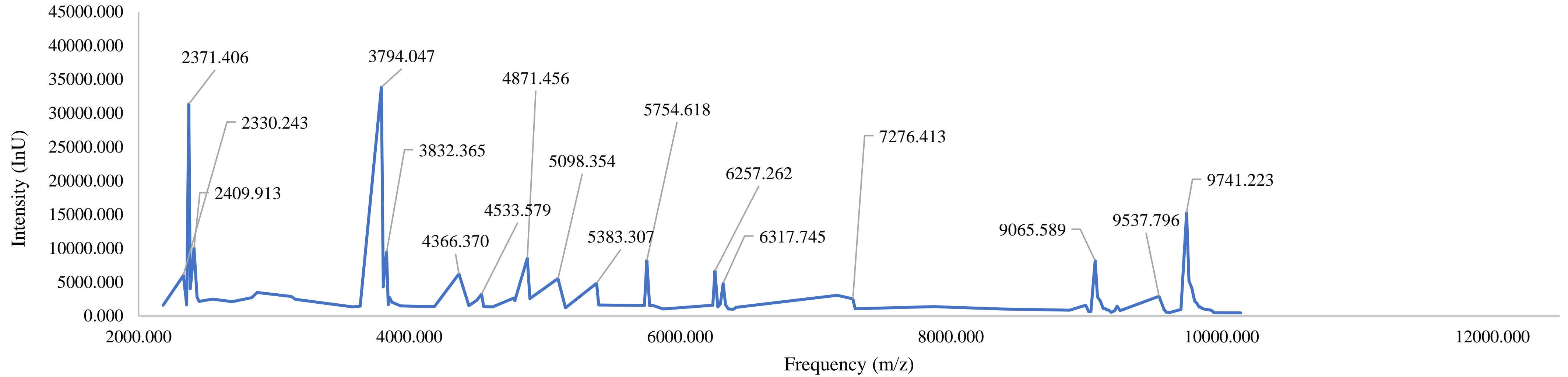


Figure 41: *E. coli* DW V2 20F
Intensity (InU)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2371.406
3794.047
4871.456
5754.618
2330.243
3832.365
5098.354
7276.413
6257.262
9741.223
2409.913
4533.579
9537.796
9065.589
4366.370
5383.307
6317.745
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

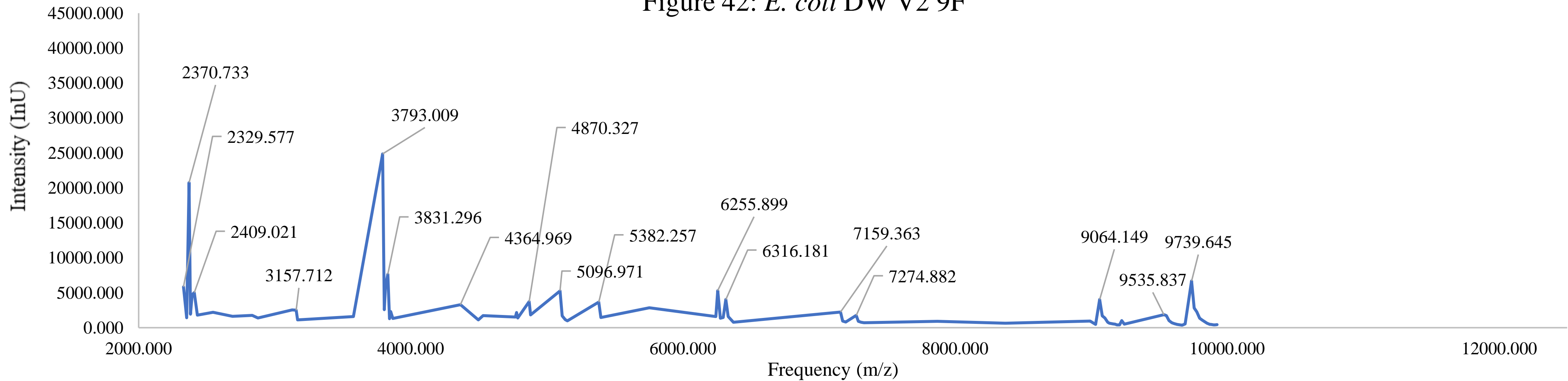


Figure 42: *E. coli* DW V2 9F
Intensity (InU)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2370.733
3793.009
2329.577
4870.327
3831.296
6255.899
2409.021
4364.969
5382.257
7159.363
9064.149
9739.645
6316.181
3157.712
5096.971
7274.882
9535.837
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

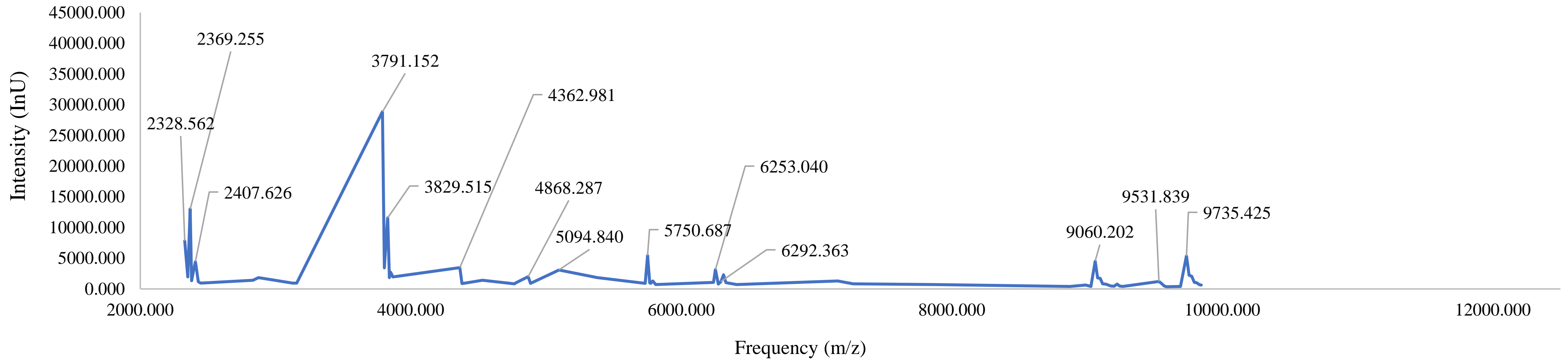


Figure 43: *E. coli* F2 10D

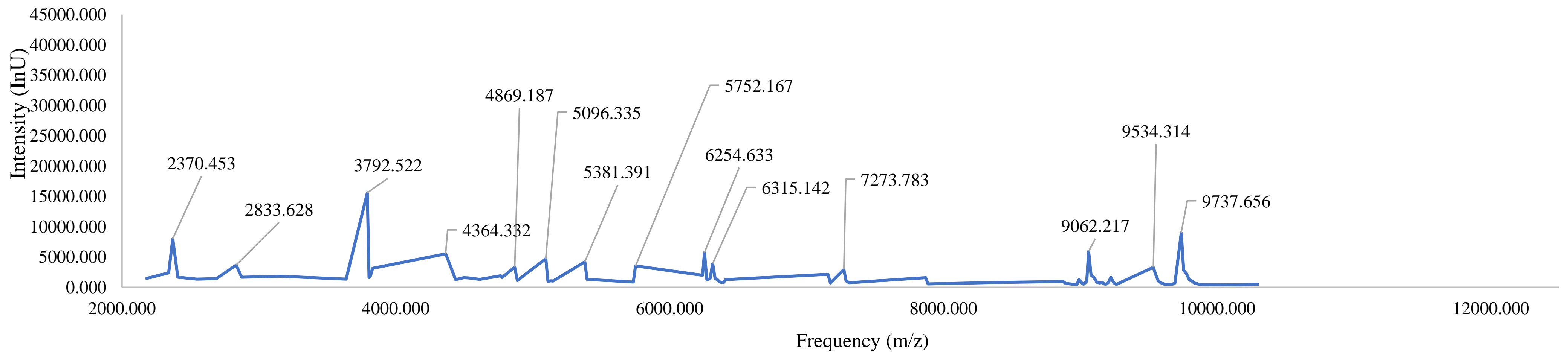


Figure 44: *E. coli* LV3 10A

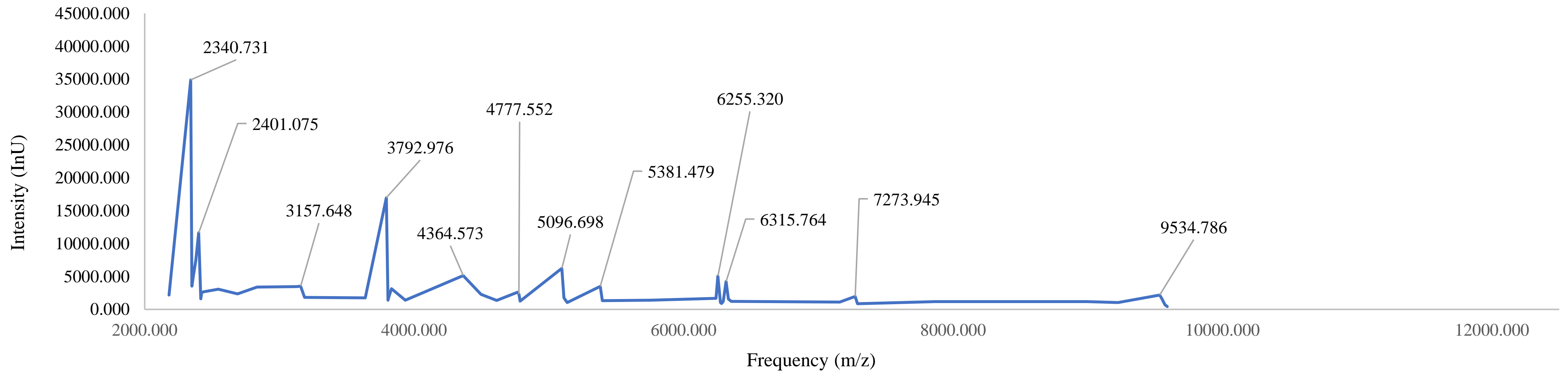


Figure 45: E. coli DW V1 21C
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2340.731
2401.075
3157.648
3792.976
4364.573
4777.552
5096.698
5381.479
6255.320
6315.764
7273.945
9534.786

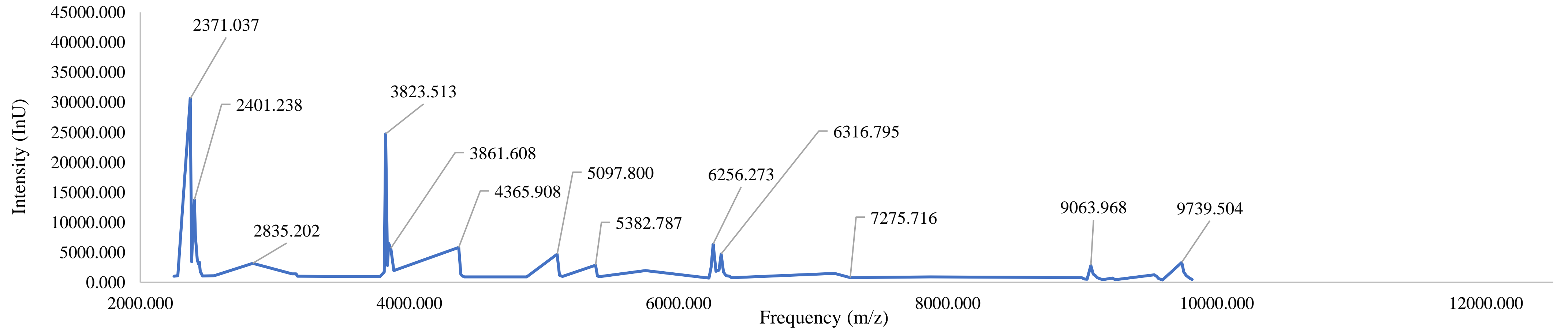


Figure 46: E. coli ESBL 3P
Intensity (InU)
Frequency (m/z)
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
2371.037
2401.238
2835.202
3823.513
3861.608
4365.908
5097.800
5382.787
6256.273
6316.795
7275.716
9063.968
9739.504

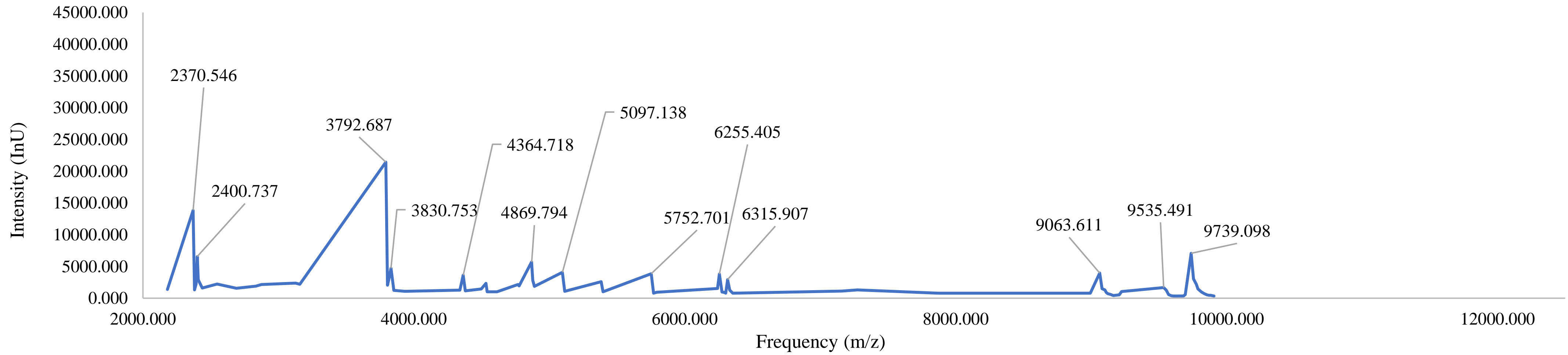


Figure 47: *E. coli* ESBL 2P
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensity (InU)
2370.546
2400.737
3792.687
3830.753
4364.718
4869.794
5097.138
5752.701
6255.405
6315.907
9063.611
9535.491
9739.098
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

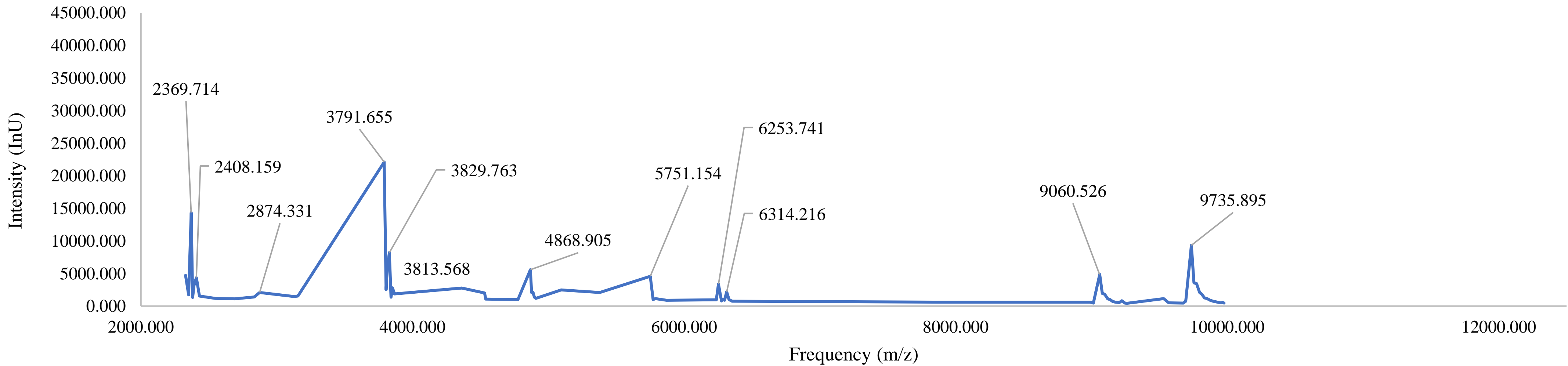


Figure 48: E. coli ESBL 8P
45000.000
40000.000
35000.000
30000.000
25000.000
20000.000
15000.000
10000.000
5000.000
0.000
Intensity (InU)
2369.714
2408.159
2874.331
3791.655
3829.763
3813.568
4868.905
5751.154
6253.741
6314.216
9060.526
9735.895
2000.000
4000.000
6000.000
8000.000
10000.000
12000.000
Frequency (m/z)

MALDI – TOF Clustered by strains

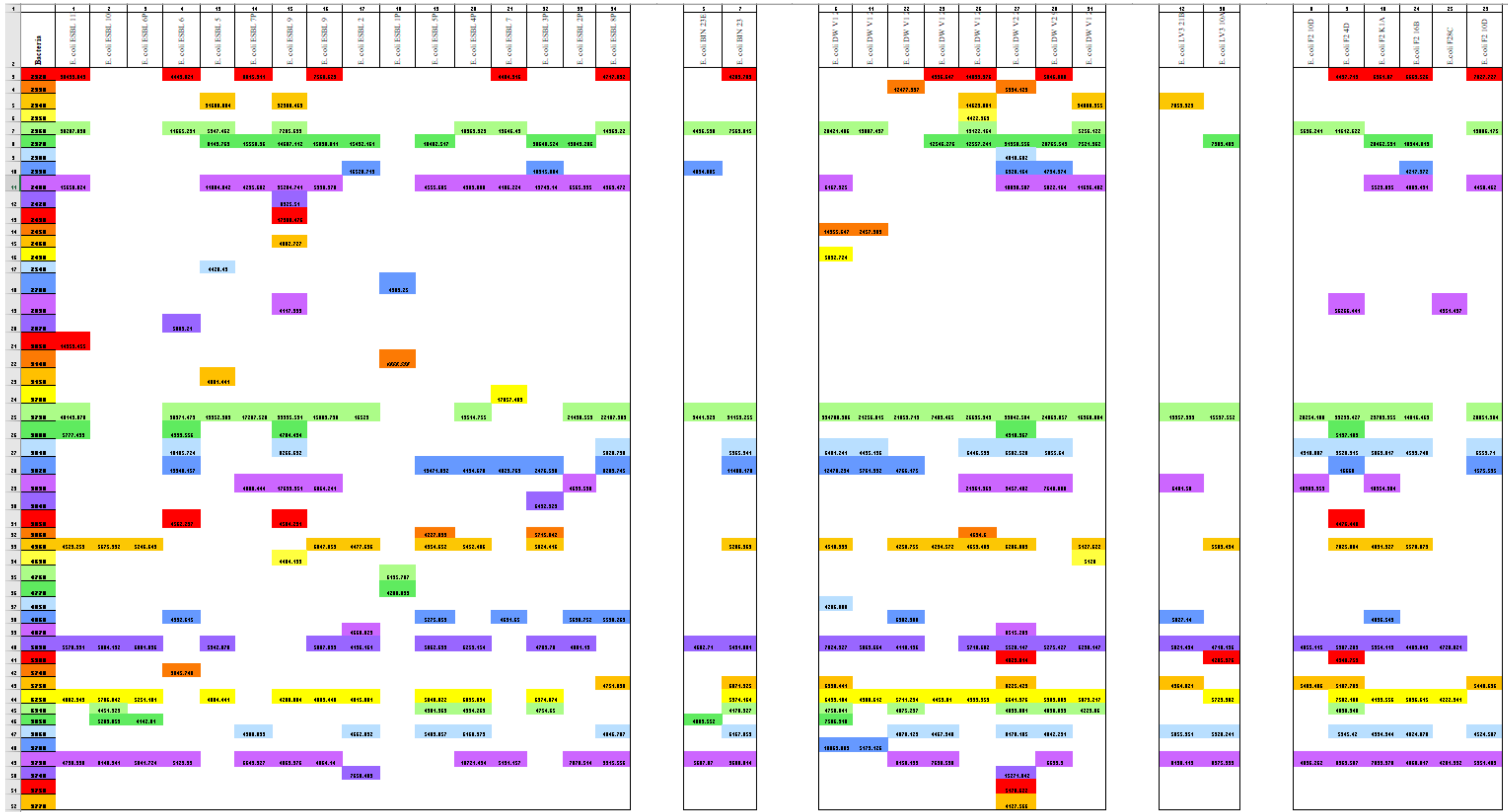


Figure 49: MALDI -TOF clusters separated by group / farm code (ESBL, BIN, DW, LV3 and F2). The individual colours show the frequency (*m/z)* cluster groups and the numbers within the colour blocks intensity values (Inu)

MALDI – TOF Clustered by similar frequency peaks

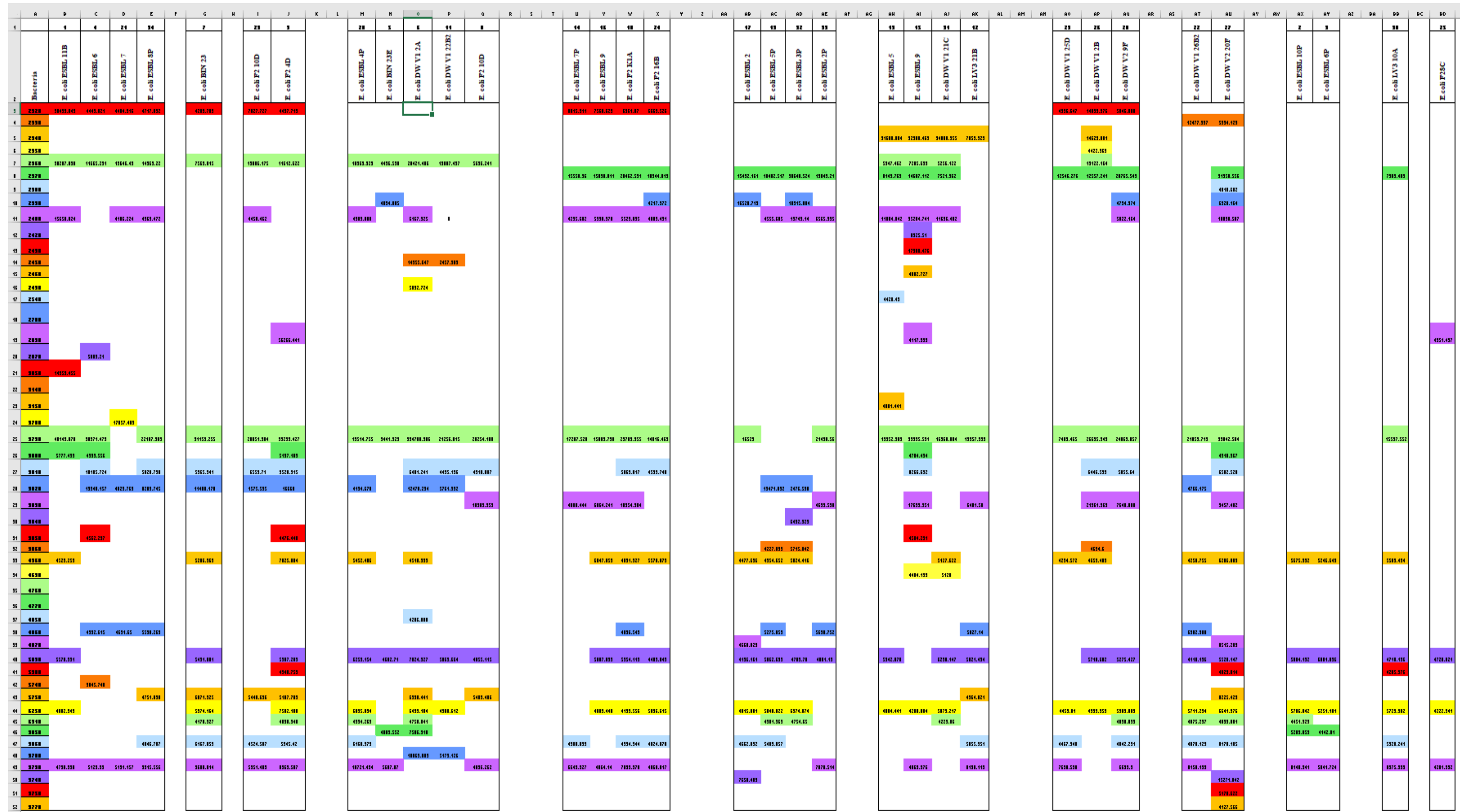


Figure 50: MALDI -TOF data shows E.coli strains separated by similar frequency *(m/z)* peaks. This shows which strains are similar to each other and therefore determine whether or not phages that infected one bacterium can also infect another bacterium with similar proteins .

Table 12: Bacteria with similar frequency peaks grouped. Phages that infected isolates are listed										
GROUP	**1**				**2**					
Bacteria	**ESBL 11P**	**ESBL 6**	**F2 10d**	**F24d**	**BIN 23E**		**ESBL 4P**	**DW 12A**	**DWV1 22B2**	**F2 10D**
Phage	HV1 P7	HV2 P4	HV4 P53	HV5 P61	HV1 P1	HV2 P5	HV4 P51	MV1 P14	MV1 P12	HV4 P53
	MV1 P15	HV2 P5	HV5 P62	HV5 P80	HV1 P7	HV1 P6	MV2 P32	HV2 P5	MV2 P16	HV5 P62
		HV1 P6	HV4 P57	HV7 P86	HV2 P4	HV2 P3				
		HV2 P3	HV5 P66	HV7 P88	HV1 P11	HV5 P80				
		HV3 P49		HV5 P65	HV2 P2	HV7 P88				
		HV1 P43		HV2 P2	HV2 P4	HV7 P86				
		HV1 P1		HV2 P3						

Table 13: Bacteria with similar frequency peaks grouped. Phages that infected isolates are listed													
GROUP	**3**					**4**					**5**		
Bacteria	**F2 16B**		**F2 K1A**	**ESBL 7P**	**ESBL 9**	**ESBL 2P**		**ESBL 2**	**ESBL 5P**	**ESBL 3P**	**DWV1 25D**	**DWV1 2B**	**DWV2 9F**
Phage	HV2 P24	HV2 P25	HV4 P54	MV2 P16		HV1 P43	HV5 P61	MV1 P15	HV1 P28	HV3 P47	HV2 P74	HV2 P5	WV2 P104
	HV1 P31	HV1 P6	HV5 P63	MV2 P18		HV3 P46	HV5 P62	HV1 P43	HV1 P31	HV1 P6	HV2 P25		WV1 P106
	MV2 P33	MV2 P35				HV3 P47	HV5 P64		HV1 P6	HV1 P43	HV4 P56		WV2 P107
	HV1 P28	MV1 P37				HV1 P6	MV2 P32		HV2 P4		HV1 P27		WV2 P108
	MV1 P14					HV4 P53	MV2 P34		HV2 P5		MV1 P14		WV1 P109
						TV1 P39			MV2 P19		MV2 P17		WV1 P110
									MV2 P20				WV1 P103
													WV2 P105

Table 14: Bacteria with similar frequency peaks grouped. Phages that infected isolates are listed						
Group	**6**		**7**		**8**	**9**
Bacteria	**DWV1 26B2**	**DWV2 20F**	**ESBL 10P**	**ESBL 6P**	**LV3 10A**	**F28C**
Phage	HV2 P76	MV3 P68	HV4 P53	HV2 P3	HV4 P55	HV1 P27
		HV6 P77	HV1 P6	HV2 P4	HV5 P64	HV1 P29
		MV3 P69		HV3 P49		HV1 P30
		HV2 P74		MV1 P23		MV2 P32
				MV2 P16		MV2 P34
				MV2 P18		MV1 P36

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