Comparative Analysis of CRISPR-Cas Systems *of Yersinia pestis* and *Escherichia coli* strains

(October 2020 – January 2022)

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Department of Mathematics and Natural Science
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January 2022

Declaration

It is hereby declared that:

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
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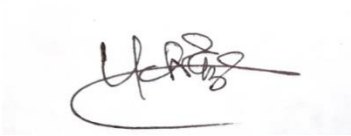

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Approval

The thesis/project titled "Comparative Analysis of CRISPR-Cas Systems *of Yersinia pestis* and *Escherichia coli* strains. (November 2020 – December 2021)" submitted by Eze Uchenna Nwabunwanne (ID:20276004) of Fall, 2020 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology on 23rd December, 2021.

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Acknowledgement

It is my pleasure to express my utmost gratitude to Almighty God for his abundant blessings, grace, mercies, and strength for timely and successful completion of my research thesis. In addition, I would like to express my profound appreciation and endless love to my beloved wife, parents, and family their courage, support, counsel and prayers to me for the accomplishment of my research studies. While undertaking this research, many people provided motivation, contributions, support, and advice that helped and guided me a lot. So, it would be an honor for me to recognize their efforts and express my gratitude and appreciation to them.

First and foremost, I wish to sincerely express my esteemed gratitude and appreciation to my thesis supervisor and Biotechnology Program coordinator, Assistant Professor Iftekhar Bin Naser, PhD for his guidance, contributions, and support towards the successful completion of my research project. More so, I remain grateful to him for his consistent effort and encouragement that inspired me to develop a strong desire and interest in research studies, especially in Molecular Biology of CRISPR Bacteriophages and Microorganisms. Indeed, it was a great honor to finish this work under his supervision.

Furthermore, I wish to express my sincere gratitude to the Chairperson, Professor A. F. M. Yusuf Haider, Academic Staff of the Mathematics and Natural Sciences Department, and the Management Staff of the University, for their supports, encouragements, contributions, as well as providing me with a fully funded scholarship opportunity to undertake this Postgraduate Master's Degree program in Biotechnology in this reputable University.

Eze Uchenna Nwabunwanne
3rd January, 2022

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Abstract:

The CRISPR-Cas system primarily refers to the clustered regularly interspaced short palindromic repeats (CRISPR) and its related protein enzymes (Cas) that confer adaptive protection against bacteriophages and other exogenous elements. The CRISPR-spacers that constitute the CRISPR-Cas systems have been reportedly found in Prokaryotes and Archea. This study analyzed and compared genome assemblies of *E. coli* and *Yersinia pestis* with the aid of bioinformatics tools (MinCED, MUSCLE alignment tool, nucleotide Basic Local Alignment Search tool (nBLAST), and prokaryotic database of the National Centre for Biotechnology Information (NCBI). The CRISPR-spacer analyses of meta-genomic sequences of 162 *Escherichia coli* and 121 *Yersinia pestis* strains showed the presence of 4 CRISPR classes (I, II, III, and IV) in *E. coli* and 3 CRISPR classes (I, II, and III) in *Y. pestis*, respectively. The result of MinCED-CRISPR analysis of CRISPR size of genome assemblies of *E. coli* and *Y. pestis* strains showed that *E. coli* CRISPR class I had the highest percentage value (51.9%) compared with *E. coli* class II (42%), *Y. pestis* CRISPR class I (40%), *Y. pestis* CRISPR class II (36%), *Y. pestis* CRISPR class III (24%), *E. coli* class III (4.8%) and *E. coli* class IV (1%). It further revealed that no CRISPR sequences were found in class IV of *Y. pestis* strains. The result of CRISPR-spacer contents also showed that the E. coli CRISPR class I showed the highest significant percentage value (54.6%) than *Y. pestis* CRISPR class I (36.93%), *Y. pestis* CRISPR class II (41.13%), *Y. pestis* CRISPR class III (21.93%), *E. coli* CRISPR class II (42.18%), *E. coli* class III (2.86%) and *E. coli* class IV (0.33%). It was observed that there was absence of CRISPR class IV in the genomic sequences of *Y. pestis* strains analyzed. The percentage distribution of exogenous spacers among *E. coli* CRISPR classes revealed that CRISPR class I showed 65.77% homologous sequence match with plasmids spacers, 51.96% bacteriophages spacers, 68.26% bacterial spacers, and 20.18% unknown target spacers, while CRISPR II had 79.82% homologous sequence match with unknown self-targets spacers, 49% bacteriophages spacers, 35% plasmids and 29.9% from other bacterial spacers available in NCBI databank. *E. coli* CRISPR class III showed no homologous spacers sequence match with unknown targets and bacteriophages but showed homologous sequence match with plasmid (0.19%) and other bacteria (1.83%). *E. coli* CRISPR class IV showed absence of homologous spacers matches against bacteriophages spacers from the NCBI databank. The spacer distribution among *Y. pestis* CRISPR classes revealed that the CRISPR class I showed a highest percentage value of homologous sequence match with 47.62% bacteriophages spacers, 38.7% plasmids, and 38.44% from other bacterial spacers, while the *Y. pestis* CRISPR class II showed spacers similarity with plasmids (25.45%) and bacteriophages (25.71%) and *Y. pestis* CRISPR III also showed percentage similarity sequence match with plasmids spacers (35.84%), bacteriophages spacers(26.67%) and 22.06% bacterial spacers available in NCBI databank. However, there was no homologous spacer sequence match contributed by unknown mobile genetic targets in all the *Y. pestis* CRISPR classes compared with the E. coli CRISPR classes. The evolutionary relationships of 22 representative strains of each bacterium were carefully selected on temporary relationship over a specific period [n < 10 years] and analyzed. The phylogenetic tree of *E. coli* revealed that 22 *E. coli* strains showed a common ancestral origin from *E. coli* BIDMC_74, and the *E. coli* BIDMC_74 strain was more closely related to *E. coli* strain IH57218 than E. *coli* str. HVH 50 and *E. coli* str. 122262 NODE_1 respectively. Phylogenetic analysis of all 22 *Y. pestis* showed a close relationship with one another, suggesting a common evolutionary relationship among them. The results obtained from this study give credence to show that the *E. coli* showed a significant CRISPR diversity than *Y. pestis*, in terms of its CRISPR class size, spacer's contents, exogenous homologous sequence matches and phylogenetic relationships among its strains. This characteristic feature showed by *E. coli* strains could be attributed to increased homologous spacer acquisition from exogenous plasmids, bacteriophages, bacteria, and unknown targeting elements. The findings further suggest that the increased CRISPR diversity observed in the *E. coli* could be associated with increased exposure of its strains to these exogenous elements than *Y. pestis* strains due to bacteriophages infection,co-evolution and conjugation with exogenous elements.

INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction:

Bacteria dominate many natural habitats, including unfavorable ones, even though they are frequently attacked by predatory viruses such as bacteriophages. Bacteriophages are bacteria-infecting viruses that are often considered the most abundant and diverse organisms in the biosphere due to their presence in nature [1]. Bacteriophages have a basic or complex particulate structure, and their genomes may encode a few to hundreds of genes, and they are made up of either genetic material (DNA or RNA) enveloped by a protein coat [2]. Bacteriophages may exist as a single organism or in combination with bacteria in nature and there are more than 1,031 bacteriophages in the globe [3, 4]. Bacteriophages are regarded as one of the most abundant living organisms in the water, and often infect 70% of bacteria in the ecosystem [5**].** Importantly, phage infects bacteria to propagate and multiply in the host organism, as well as reproduce within the host by integrating its genome into the host organism's cytoplasm [5]. Following the injection of their genetic material into the bacterium, they multiply and integrate their genetic material into the host organism, making them a useful molecular tool for genome editing and genetic manipulation [6,7]. The infiltration of bacteriophages into the host organism as well as the integration of its genetic material led to the evolution of a protective and adaptive immunity by host organism [7]. The evolution of the CRISPR system in the host organism provides adaptive immunity to the bacteria and other microbes against bacteriophages' invasion into the host [8]. CRISPRs have been shown experimentally to protect *Streptococcus thermophilus* and *Staphylococcus epidermidis* against phages and plasmids, respectively [9]. Also, it has been shown that CRISPR sequences are found, in approximately 50% of sequenced bacterial genomes and nearly 90% of sequenced archaea [10].

CRISPR constitute a family of DNA sequences found in genomes of prokaryotic organisms usually bacteria and archaea, and their sequences are derived from DNA fragments of bacteriophages that had previously infected the prokaryotes [11]. Studies have shown that CRISPR provides innate adaptive immunity to several bacteria and archaea, through the use of a microbial protective enzyme**,** *Cas* endonucleases (nucleases) that are involved in the anti-phage defense system. CRISPRs have guide sequences used to detect and destroy DNA sequences from similar bacteriophages during subsequent infections [12,13]. These CRISPR sequences play a key role in the antiviral (anti-phage) defense system of prokaryotes, hence confers a form of acquired immunity to the host organism [14]. For instance, Cas 9 (CRISPR-associated protein 9) is

an enzyme that uses CRISPR sequences as a guide to recognize and cleave specific strands of DNA which are complementary to the CRISPR sequence found in the bacteriophages and other exogenous elements [15]. The Cas9 enzymes together with CRISPR sequences form the basis of a technology known as CRISPR-Cas9 tool which can be used to edit genes within organisms [16]. Several studies have shown that the repeat nucleotide sequences, otherwise termed "Clustered Regularly Interspaced Short Palindromic Repeats regions (CRISPRs)" are characterized by short, and perfectly conserved elements consisting of 20 to 40 nucleotide base pairs separated by spacer sequences [17].

In several bacteria anti-phage defenses, it has been shown that CRISPR–Cas systems could be used to detect and destroy foreign nucleic acids and exogenous elements through the use of CRISPR RNAs (crRNAs) and Cas nucleases [18,19]. In addition, these CRISPRs have several functions that are directly involved in DNA rearrangement and replication, host cell defenses, and guide RNA (gRNA) control, and most bacterial cells have developed numerous means to survive by fending off phage viruses and other exogenous elements during virulent conditions through the recognition of their sequences and breakdown of their genetic materials by the use of their CRISPR-cas enzymes [20]. Bacteria also resist phages, due to presence of phage-inducible chromosomal islands (PICI), in such a way to defend against phage infection and invasion of the bacterial chromosome upon phage infection, replicates, and interferes with phage reproduction [21].

Notably, the CRISPR-Cas system has been broadly classified into two broad CRISPR classes, six distinct CAS subtypes (I–VI), and dozens of cas enzymes from different *CAS* genes, with diverse mechanisms of actions [22,23]. For instance, the Class 1 system (subtypes: I, III, and IV) encode multi-subunit effector complexes, and the Class 2 system (subtypes: II, V, and VI) relies upon a single subunit to destroy nucleic acid invaders [24]. Several notable research attempts have explicitly elucidated and analyzed the functional mechanism of the CRISPR-cas adaptive immunity in the host organism and discovered that the CRISPR arrays are separated by different short sequences known as "*spacers"* [25]. However, the functional mechanisms have showed that the CRISPR–Cas immunity occurs in three main steps namely: adaptation, CRISPR RNA (crRNAs) biogenesis, and interference. During the adaptation stage, short (30–40 nucleotides)

invader-derived sequences called *"spacers"* are captured and integrated into CRISPR loci in between partially palindromic DNA repeats of similar length [25]. During crRNA biogenesis, the repeat–spacer array is transcribed into a long precursor crRNA, which is further processed to liberate mature crRNAs that specifies a single target. During interference, crRNAs combine with one or more *CAS* proteins to form an effector complex that recognizes and degrades nucleic acids (*protospacers*) that are complementary to the crRNA [26]. The crRNA biogenesis and interference stages constitute the defense phase of CRISPR–Cas immunity, and all CRISPR–Cas systems adhere to this general pathway [27]. The three basic stages appear to be common to all CRISPR systems, CRISPR loci, and the proteins that mediate each stage of adaptive immunity are remarkably diverse [28].

1.1 Literature Review:

In 1896, Ernest Hanbury Hankin reported that some particles in the waters of the Ganges and Yamuna rivers in India had a marked antibacterial action against cholera and it could pass through a very fine porcelain filter [29]. In 1915, Frederick Twort, a British bacteriologist and superintendent at the Brown Institute of London discovered a small agent that infected and killed bacteria [30]. He believed the agent must be one of the following: *a stage in the life cycle of the bacteria, the enzyme produced by the bacteria themselves, or a virus that grew on and destroyed the bacteria* [31]. However, Twort's research was interrupted by the onset of World War I, due to shortage of funding, and his works led to the discovery of antibiotics [32]. Independently, French-Canadian Microbiologist Félix d'Hérelle, who worked at the Pasteur Institute in Paris, announced on September 3, 1917, that he had discovered "*an invisible, antagonistic microbe of the dysentery bacillus*"[33]. Even though, d'Hérelle thought there was no question as to the nature of his discovery, but reported his observation: "*In a flash, I had understood: what caused my clear spots was an invisible microbe - a virus parasitic on bacteria* [34]." d'Hérelle named the virus as "*bacteriophages*", implying a bacteria-eater (Greek phage in meaning "to devour")[35]. He also recorded a dramatic account of a man suffering from dysentery who was restored to good health by the bacteriophages [36]. Notably, d'Herelle was known to have conducted much research into bacteriophages and later introduced the concept of phage therapy [37].

The classification of viruses in the early 1940s by Lwoff, Horne and Touiner recognized and took

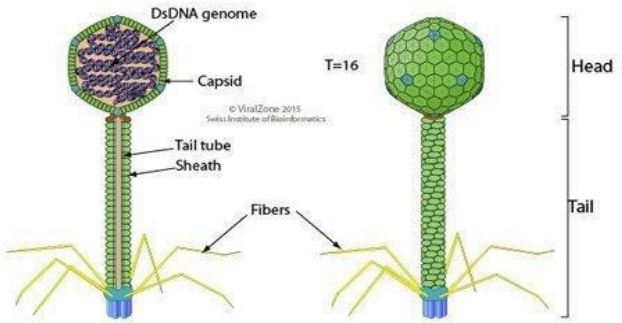
into account of the viral nature of bacteriophages under the electron microscope, and classified them into groups based on their morphologies and type of nucleic acid [38]. Later, Lwoff, Horne, and Tournier published a list of the order of the classification of prokaryotic viruses (Table 1.1). The phages were classified into six groups based on their morphologies and types of nucleic acid by the International Committee on Taxonomy of Viruses (ICTV) (as shown in Table 1.1). Some of the major classes of these Bacteriophages include *Urovirales* for tailed phages: *Microviridae* (icosahedron), *Inoviridae* (filamentous), and ΦX-type phages [39].

Table 1.1: ICTV CLASSIFICATION OF PROKARYOTIC (BACTERIAL AND ARCHAEAL) VIRUSES

Order	Family	Morphology	Nucleic acid	Examples
Belfryvirales	*Turriviridae*	Enveloped, isometric	Linear dsDNA	-
Caudovirales	*Ackermannviridae*	Nonenveloped, contractile tail	Linear dsDNA	-
	Myoviridae	Nonenveloped, contractile tail	Linear dsDNA	*Bacteriophages:T4, Mu, P1, P2*
	Siphoviridae	Nonenveloped, noncontractile tail (long)	Linear dsDNA	*Lambda phage(λ) Phage T5, Bacteriophage HK97, Enterobacteria phage (N15)*
	Podoviridae	Nonenveloped, noncontractile tail (short)	Linear dsDNA	*T7 (Bacteriophage T7) T3 (Bacteriophage T3) Bacillus phage phi 29(Φ29), Enterobacteria phage 22(P22)*
Halopanivirales	*Sphaerolipoviridae*	Enveloped, isometric	Linear dsDNA	-
Haloruvirales	*Pleolipoviridae*	Enveloped, pleomorphic	Circular ssDNA, circular dsDNA, or linear dsDNA	-
Kalamavirales	*Tectiviridae*	Nonenveloped, isometric	Linear dsDNA	-
Levivirales	*Leviviridae*	Nonenveloped, isometric	Linear ssRNA	*Bacteriophages MS2, Bacteriophages Qβ*
Ligamenvirales	*Lipothrixviridae*	Enveloped, rod-shaped	Linear dsDNA	Acidianus filamentous virus 1
	Rudiviridae	Nonenveloped, rod-shaped	Linear dsDNA	Sulfolobusislandicus rod-shaped virus 1
Mindivirales	*Cystoviridae*	Enveloped,	Segment	*Pseudomonas virus phi*

		spherical	ed dsRNA	(Φ6)
Petitvirales	*Microviridae*	Nonenveloped, isometric	Circular ssDNA	*Bacteriophages Phi ΦX174*
Tubulavirales	*Inoviridae*	Nonenveloped, filamentous	Circular ssDNA	*M13 bacteriophages*
Vinavirales	*Corticoviridae*	Nonenveloped, isometric	Circular dsDNA	*Psueodoalteromonas virus PM12*
Unassigned	*Ampullaviridae*	Enveloped, bottle-shaped	Linear dsDNA	-
	Bicaudaviridae	Nonenveloped, lemon-shaped	Circular dsDNA	-
	Clavaviridae	Nonenveloped, rod-shaped	Circular dsDNA	-
	Finnlakeviridae		dsDNA	*FLiP(Flavobacterium virus)*
	Fuselloviridae	Nonenveloped, lemon-shaped	Circular dsDNA	-
	Globuloviridae	Enveloped, isometric	Linear dsDNA	-
	Guttaviridae	Nonenveloped, ovoid	Circular dsDNA	-
	Plasmaviridae	Enveloped, pleomorphic	Circular dsDNA	-
	Portogloboviridae	Enveloped, isometric	Circular dsDNA	-
	Spiraviridae	Nonnveloped, rod-shaped	Circular ssDNA	-
	Tristromaviridae	Enveloped, rod-shaped	Linear dsDNA	-

Phages are divided into several categories depending on their shape, sizes, and genetic make-up of single nucleic acid (DNA or RNA) enclosed by a protein capsid coat [40]. The great majority of phages usually have protein tails that allow them to recognize a receptor on the surface of the host bacterium [41]. In 1967, Bradley recognized six basic phages, and further classified them into three groups as tailed, filamentous and icosahedral phages with either single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA)[42].Presently, newly discovered bacteriophages are classified into these groups, based on their size and shapes namely: Icosahedron bacteriophages, Filamentous bacteriophages, and Complex bacteriophages (as shown in Figure 1.1).



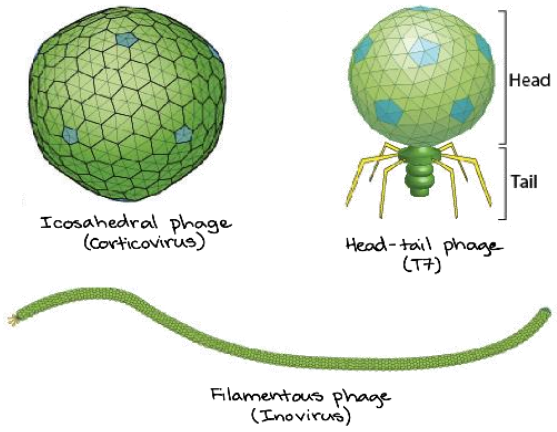


Figure1.1: Structure of T_4 bacteriophage, Corticovirus (Icosahedron) and Inovirus (Filamentous).

i. **Icosahedron bacteriophages**: this type of bacteriophage has a spherical shape, with twenty triangular facets, and the smallest size of 25nm in diameter.

ii. **Filamentous bacteriophages:** They are made up of long tubes formed by capsid protein and assembled into the helical structure with about 900nm in diameter size.

iii. **Complex bacteriophages:** They have icosahedral heads attached to helical tails fibers and base plates.

The Life cycle of Bacteriophages is classified into two groups namely: **lytic (virulent) and lysogenic (temperate) bacteriophages,** according to their biological cycles [43]. Bacteriophages are infectious to bacteria, due to the presence of receptors on their surfaces which enable bacteria to bind onto the phage as well as identify their specific host organisms [43]. Bacteriophages bind to their host's cell receptors by adsorption and inject their genetic material into the host cell. The significant distinction between the lytic and lysogenic phage cycles lies in the fate of bacteria [44]. In the lytic cycle, lytic phages take over the bacterial replication machinery by reproducing a new phage virus (progeny) within its host. As a result of the

reproduction of multiple copies of these new phages that form a critical *mass* which triggers lysing of the bacteria cell wall, and the release of new phage viruses, and beginning of another lytic cycle [45]. The formation of critical mass (or burst size) mostly depends on several factors including specific phage characteristics, bacteria-infected by the phage, and the environment within which the phage-bacteria interaction occurs [46].

Contrastingly, lysogenic phages involve the integration of the genetic material of lysogenic phages into the host which results in the formation of an entity called "prophage" [47]. The prophage is involved in the vertical transmission of genetic information of the phage virus to newly formed bacteria daughter cells by cell division, and expression of viral genes and proteins [48]. Less commonly, the genetic material of lysogenic phage does not integrate itself into the host bacterial chromosome, but remains in the intracellular as a separate plasmid, until it becomes transferred to new bacterial cells. Under exceptional circumstances, it has been reported that environmental stress may likely induce a transition from a lysogenic cycle into a lytic cycle [49]. As a result of their ability to cause bacterial cell lysis, lytic phages are often used in phage therapy, because of their unique characteristics, while lysogenic phages confer anti-microbial resistance [50].

Notably, the CRISPRs consist of a diverse family of DNA repeats that all share a common architecture. Each CRISPR locus consists of a series of short repeat sequences typically containing 23–47 base pairs long separated by unique spacer sequences of a similar length (as shown in Figure 1.2**)** [50]. The repeat sequences within a CRISPR locus are conserved, and other repeat sequences in different CRISPR loci can vary in both sequence and length [51].

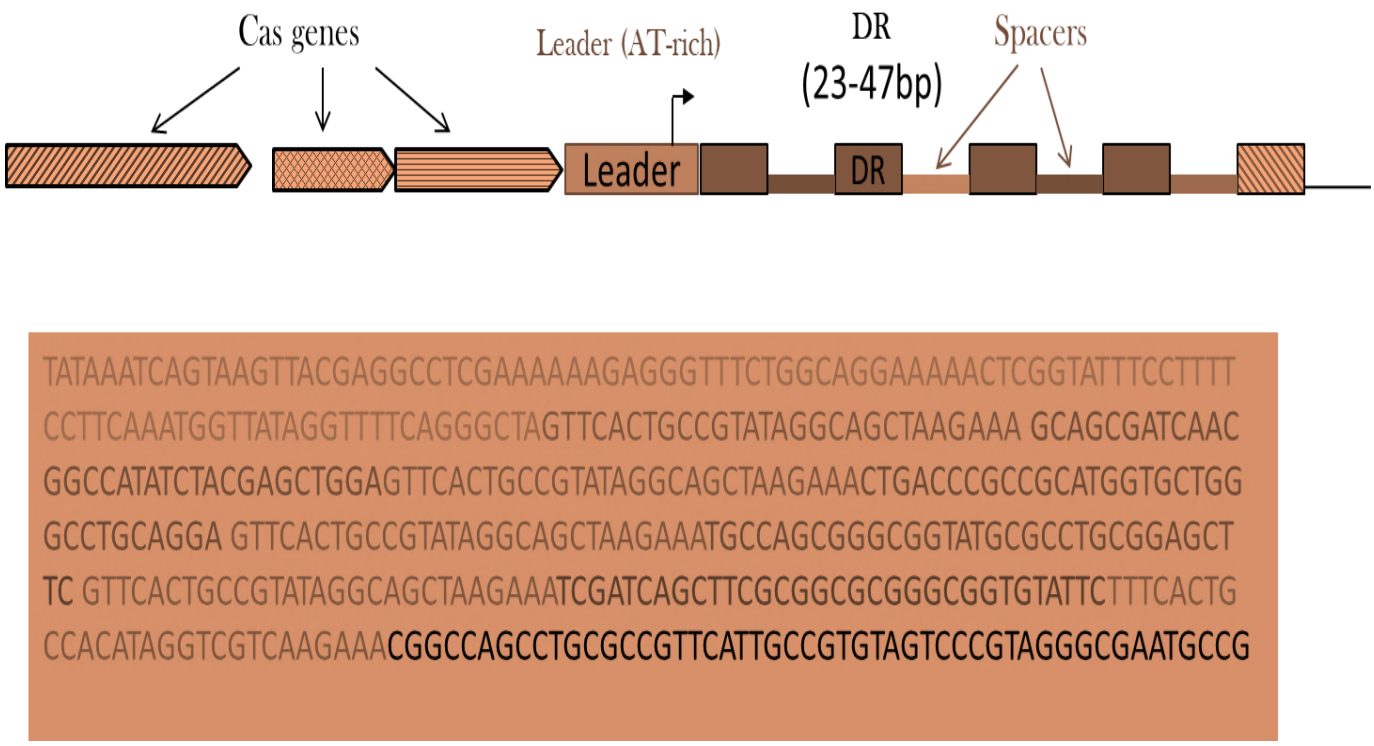


Figure 1.2: Structural description and components of CRISPR-cas system

The CRISPR repeat clusters were initially numbered 1 to 12, whereas the CAS systems were first designated after a representative organism, using a three-letter code, and each *CAS* gene was assigned a number according to its position in the *CAS* gene cluster (e.g., cse1, cse2) [41]. The *CAS* genes in the other systems were named using a similar strategy, while some of the *CAS* gene families were later determined to be orthologous and renamed using a "clusters of orthologous groups" classification scheme [42]. The diversity of CAS genes and their association with different CRISPR repeat clusters had also made it difficult to arrive at common nomenclatures that are easy to understand, but these pioneering phylogenetic studies were critical in establishing a basis for biochemical and mechanistic investigation [43]. The CAS gene and CRISPR repeat phylogenies are now combined in a novel categorization method [43]. Three major forms of CRISPR/Cas systems have been identified using this method, and each of these major types has been described. In addition to the leader sequence, comparative research studies have identified a range of CAS genes, which are often located close to a CRISPR locus. Initially, four CAS genes were found in genomes containing CRISPRs, but as genome sequences grew larger and more sophisticated search methods were developed, a total of 45 gene families linked with CRISPRs have been revealed [44]. Six of these *CAS* genes (*cas1–cas6*) are widely conserved and

are considered core *CAS* genes, but only *cas1* and *cas 2* are universally conserved in genomes that contain CRISPR loci [45]. *Cas1* is a hallmark of this immune system, and phylogenetic analysis of *cas1* sequences suggests several distinct versions of CRISPR systems exist [46]. Each of these different phenotypes is defined by a unique composition and conserved arrangement of *CAS* genes [47]. Remarkably, this *CAS* gene-based classification appears to correlate well with a CRISPR repeat–based classification, suggesting that the *Cas* proteins interact with specific sets of CRISPR loci [47]. CRISPR-Cas systems have been classified into three major types, namely type I, type II, and type III, and 12 subtypes, given their genetic content, structural and functional differences [48]. The core defining feature of CRISPR-Cas types and subtypes are the CAS genes and the proteins they encode, which are highly genetically and functionally diverse, illustrating the many biochemical functions that they carry throughout the different steps of CRISPR-mediated immunity. Noteworthy, the RNA recognition motif is widespread in many Cas proteins, and most of the Cas families of proteins carry functional domains that interact with nucleic acids, such as DNA binding, RNA binding, helicase, and nuclease motifs [49]. Genetically, cas1 and cas2 universally occur across types and subtypes, whereas cas3, cas9, and cas10 have been defined as the signature genes for type I, type II, and type III, respectively. Phylogenetic analysis has shown that type II systems have solely been identified in bacteria, thus far, and there is a bias for type I systems in bacteria and type III systems in archaea and hyperthermophiles [48,49].

The molecular mechanism of CRISPR-Cas system is categorized into three functional phases (as shown in Figure 1.3) and they include; **immune specificity adaptation, crRNA expression and maturation, and target interference.** At one end of the CRISPR locus, fragments of foreign DNA are integrated during the adaption step. Although, cas1 and cas2 are known to be required for adaptation, and the process of spacer acquisition is yet unclear [48,52]. The CRISPR array is transcribed into a lengthy precursor crRNA during the expression stage, which is then cleaved in the repetitions by specialized *CAS* proteins or RNase III, and occasionally trimmed to generate mature crRNAs. Subsequently, the *CAS* protein complex is loaded with these short guide RNAs [49].

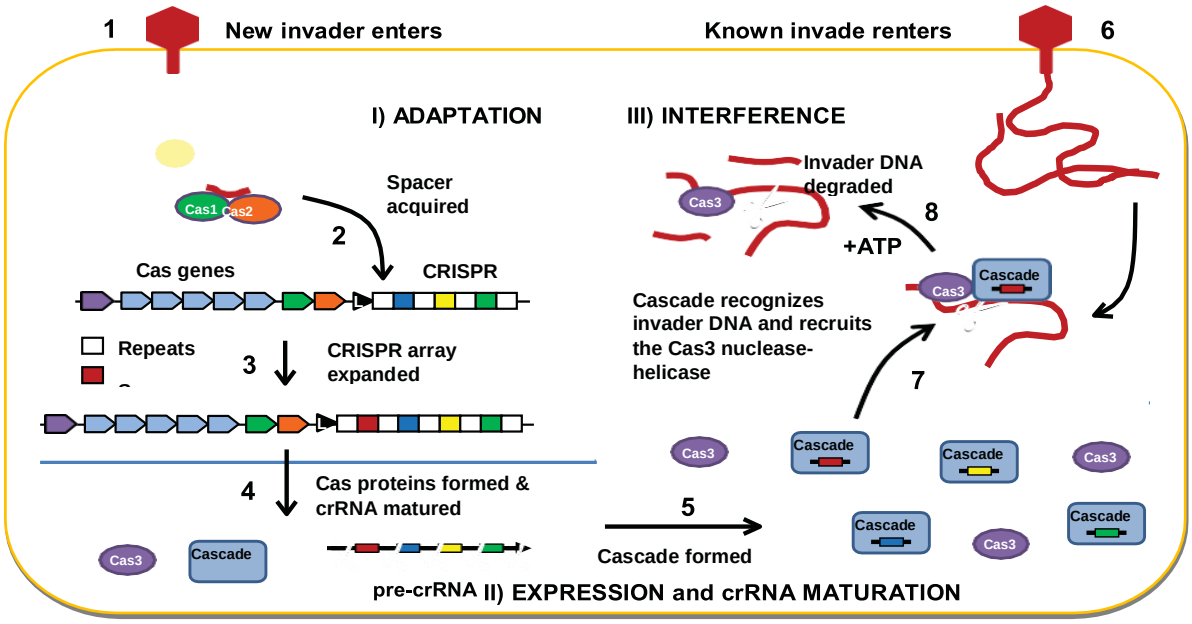


Figure 1.3: Three Functional Steps of CRISPR-Cas Mechanism

Yersinia pestis is a causative agent of plague and exhibits multi-host and multi-vector pathogenic characteristics [50]. Over 200 species of this organism live in wild rodents as host organisms and over 80 species of fleas as vectors. The disease caused by *Y. pestis*, is a zoonotic infection which is regarded as one of the most devastating infections in human history and the disease is transmitted to humans from natural rodent reservoirs, usually through the bite of an infected flea [50]. Different hosts and vectors have their specific ecological landscape and different levels of susceptibility to the organism. The classification of *Yersinia pestis* is shown in Table 1.4 below.

Table 1.4: Scientific classification of *Yersinia pestis*

Domain:	**Bacteria**
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacterales
Family:	Yersiniaceae
Genus:	*Yersinia*
Species:	*Y. pestis*

In addition, the survival of the bacteria in the soil is likely to contribute to the long-term persistence of *Y. pestis*. During its expansion and adaptation to new niches, *Y. pestis* undergoes

genetic variations, some of which may help overcome natural selective forces. These variations may be used as markers to reconstruct the historical spread of the plague. Because of its importance in human history, many investigations have aimed at deciphering the evolution of this major pathogen [50,51]. In 1894, Alexandre Yersin identified this organism based on its biochemical characteristics and further employed a molecular typing technique to classify Y. pestis strains. Based on these characteristics, it further constituted a strong phylogenetic signal that has been used to identify and classify typical human pathogenic *Y. pestis* (subspecies pestis in Russian nomenclature) into three *biovars, bv. Antiqua, bv. Medievalis and Orientalis bv*.[50].

Escherichia coli, commonly known as *E. coli* is a Gram-negative, facultative anaerobe and rod-shaped coliform bacteria found in the lower intestine of warm-blooded animals (endotherms) [48, 50]. Notably, the scientific classification of *E. coli* bacterium is shown in Table 1.5 below.

Table 1.5: Scientific classification of *Escherichia coli*

Domain	**Bacteria**
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacterales
Family:	Enterobacteriaceae
Genus:	*Escherichia*
Species:	*E. coli*

Although most *E. coli* strains are innocuous and categorized into different serotypes (EPEC, ETEC, and others) that may cause acute food poisoning and food contamination episodes that results to food-borne diseases. E. coli is the most studied prokaryotic model organism and a key species in biotechnology and microbiology, where it has served as the host organism for the majority of recombinant DNA research. Several pathogenic E. coli groups cause disease in humans and animals, such as diarrheagenic E. coli and extra-intestinal pathogenic E. coli (ExPEC), which cause sickness outside of the GI tract. Diarrheagenic E. coli that cause human sickness have been categorized based on particular sets of virulence genes they contain and the features of the disease they produce [47,48]. These pathotypes include enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC), enteroaggregative E. coli (EAEC), Shiga toxin-producing E. coli (STEC), diffusely adherent E. coli (DEAC), and adherent

invasive E. coli (AIEC) that have been linked to Crohn's disease[34]. There are hybrid pathotypes, such as enteroaggregative hemorrhagic E. coli (EHEC), which have both STEC and EAEC-associated virulence genes. In 2011, EAHEC serotype O104:H4, an EAEC that acquired the phage carrying the Shiga toxin gene of STEC, produced a major epidemic that resulted in sickness in over 3800 people and 54 fatalities [48]. Certain E. coli serotypes, such as STEC O157:H7 and O103:H21 are frequently linked with certain pathotypes, such as enterohemorrhagic E. coli (EHEC). As a result, pathogenic E. coli is a genetically diverse family of bacteria that is continuously evolving [51, 52].

The aim of this study is to analyze and compare CRISPR-cas systems of *Yersinia pestis and Escherichia coli* strains using bioinformatics tools.

1.2 Specific objectives:

i. To identify and analyze CRISPR and spacer compositions of meta-genomic sequences of *Yersinia pestis* and *Escherichia coli* strains.

ii. To determine and compare the pattern of exogenous spacer distributions of *Yersinia pestis* and *Escherichia coli* strains

iii. To conduct phylogenetic analysis and identify evolutionary relationships between *Yersinia pestis* and *Escherichia coli* strains.

1.3 Expected outcome

i. The pattern of exogenous spacer acquisition and distribution in the CRISPR classes of *Yersinia pestis* and *Escherichia coli* strains would be determined.

ii. The phylogenetic relationships of CRISPR of selected representative strains of both bacteria would be analyzed and established.

iii. CRISPR size and spacer sequence contents of the genome assemblies of the bacteria would be analyzed and determined.

iv. CRISPR types/classes of both bacterial species would be identified.

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

Generally, bioinformatics tools are often used to analyze and compare genetic and genomic data of prokaryotes. It provides adequate information and insight on the evolutionary relationships of prokaryotes and their molecular biology.

Bioinformatics software tools have been vastly used to identify and analyze sequences of genomes and genes of microbes. Recently, advanced bioinformatics tools have been developed to detect and analyze CRISPR and spacers sequences of microbial genomes, and to determine their evolutionary and phylogenetic relationships. These bioinformatics tools used for this study include Prokaryotic database of the National Centre for Biotechnology Information (NCBI), web-based repositories of all prokaryotes' genome database, MinCED-Linux operating software tool, and relevant microbial web servers.

In this study, NCBI databank, nBLAST tool, MUSCLE alignment tool, MinCED-CRISPR tools and online phylogeny server were used to conduct the CRISPR-cas analysis of *E. coli and Y. pestis* strains.

2.1 BIOINFORMATICS TOOLS:

2.1.1 National Center for Biotechnology Information (NCBI) PROKARYOTIC DATABASE / SERVER

NCBI prokaryotic database served as a major source of data for genomic "FASTA" nucleotide sequences of *Escherichia coli* and *Yersinia pestis* strains. The first task and procedure undertaken were to specifically search and download complete genome assemblies of available "FASTA" nucleotide sequences of *E coli* and *Yersinia pestis* strains separately from NCBI web-based prokaryotic database.

The web-based prokaryotic database of the National Center for Biotechnology Information (NCBI) contains repositories of genomic nucleotide sequences, and annotated information of Bacterial strains. NCBI online prokaryotic repository is a collection or assembly of genomes, genes, taxonomic classification of bacterial strains, and archaea. Bacterial genome sequencing started by an approach made on genome analysis through sequencing and assembly of unselected pieces of DNA to get the complete nucleotide sequence of the genome from the whole chromosome

in the year 1995 which led to a promising breakthrough in microbiology and infectious disease research.

In addition, the National Centre for Biotechnology Information advances science and health by providing access to biomedical and genomic information. NCBI has a multi-disciplinary research group that consists of computer scientists, molecular biologists, mathematicians, and biochemists, research physicians concentrating on basic and applied research in computational molecular biology. NCBI prokaryotic database contains a total number of 12,000 bacterial genome sequences.

2.1.2 MINCED (Mining CRISPRs in Environmental datasets) - LINUX SOFTWARE TOOL

MinCED is a program used to find Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) in full genomes or environmental datasets such as assembled contigs from metagenomes. It identifies CRISPRs in raw short read data, in the size range of 100-200bp and this software package can be sourced from the Crass portal (https://github.com/ ctskennerton/ Crass). The MinCED software tool runs from the command-line and was derived from CRT (http://www.room220.com/crt/).

Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) are a novel type of direct repeat found in a wide range of bacteria and archaea. CRISPRs work by defending their hosts against invading extra-chromosomal elements such as viruses. The CRISPR arrays are identified using MinCED (mining CRISPRs in environmental datasets), a derivative of the CRISPR Recognition Tool that is more conservative in repeat calling and allows more flexible user outputs.

Custom code determines the orientation of the repeats, generates the consensus repeat sequences, and returns the number of repeats by indicating the size of the array. After the identification of CRISPR loci, the types and subtypes are assigned by using the presence or absence of genes, detecting multiple systems in a genome, and identifying the missing repeats and CAS proteins it determines the completeness of the system.

2.1.3 MULTIPLE SEQUENCE COMPARISON BY LOG EXPECTATION (MusCLE) TOOL

MusCLE(Multiple Sequence Comparison by Log Expectation) is a program for creating multiple alignments of amino acid or nucleotide sequences. The MUSCLE web-based tool is used for multiple sequence alignment (MSA) of three or more biological sequences, generally a protein, DNA, or RNA.

Multiple Sequence Alignment program has an algorithm for finding regions of similarity between

biological sequences through comparing nucleotide or protein sequence from the databases and calculates the statistical significance, one of the most widely used bioinformatics programs for sequence searching. In many cases, the input set of query sequences are assumed to have an evolutionary relationship by which they share a linkage and are descended from a common ancestor.

From the resulting MSA, sequence homology can be inferred, and phylogenetic analysis can be conducted to assess the sequences' shared evolutionary origins. It provides a range of options to the user for a better choice of optimizing accuracy, speed, or some compromise between the two. Default parameters are those that give the best average accuracy.

Some published tests show that MUSCLE can achieve both better average accuracy and better speed than CLUSTALW or T-Coffee, depending on the chosen options. MUSCLE enables high-throughput applications to achieve average accuracy comparable to the most accurate tools previously available, which we expect to be increasingly important for in sequence data.

MUSCLE has been integrated into DNASTAR's Lasergene software, Geneious, and MacVector and is available in Sequencher, MEGA, and UGENE as a plug-in. MUSCLE is also available as a web service via the European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI).

2.1.4 BASIC LOCAL ALIGNMENT SEARCH TOOL (nBLAST)

In bioinformatics, **BLAST** (**basic local alignment search tool**) is an algorithm and program for comparing primary biological sequence information, such as the amino-acid sequences of proteins or the nucleotides of DNA and/or RNA sequences.

The 'BLAST' searches compare a subject protein or nucleotide sequence (called a query) with a library or database of sequences and identify database sequences that resemble the query sequence above a certain threshold.

For example, following the discovery of a previously unknown gene in the mouse, a scientist will typically perform a BLAST search of the human genome to see if humans carry a similar gene; BLAST will identify sequences in the human genome that resemble the mouse gene based on similarity of sequence.

This program, given a DNA query, returns the most similar DNA sequences from the DNA database that the user specifies. BLAST can be used for several purposes. These include

identifying species, locating domains, establishing phylogeny, DNA mapping, and comparison.

Uses of nucleotide BLAST Tool

- **By identifying species with** the use of BLAST, you can correctly identify a species or find homologous species. This can be useful, for example, when you are working with a DNA sequence from an unknown species.
- **Locating domains**

  When working with a protein sequence you can input it into BLAST, to locate known domains within the sequence of interest.
- **Establishing phylogeny**

  Using the results received through BLAST you can create a phylogenetic tree using the BLAST web page. Phylogenies based on BLAST alone are less reliable than other purpose-built computational phylogenetic methods, so should only be relied upon for "first pass" phylogenetic analyses.
- **DNA mapping**

  When working with a known species and looking to sequence a gene at an unknown location, BLAST can compare the chromosomal position of the sequence of interest, to relevant sequences in the database(s). NCBI has a "Magic-BLAST" tool built around BLAST for this purpose.
- **Comparison**

  When working with genes, BLAST can locate common genes in two related species and can be used to map annotations from one organism to another.

2.1.5 PHYLOGENY WEBSERVER TOOL

Phylogeny.fr is a web-designed program that operates on a web interface (www.phylogeny.fr) used to conduct phylogenetic studies of biological sample data. It has a high-performance platform that transparently chains programs relevant to phylogenetic analysis in a comprehensive and flexible pipeline.

Although phylogenetic aficionados will be able to find most of their favorite tools and run sophisticated analyses. The primary aim of Phylogeny.fr web- designed program is to assist biologists with no experience in phylogeny in robustly analyzing their data.

The Phylogeny.fr platform offers *a phylogeny pipeline* that can be executed through **three main modes:**

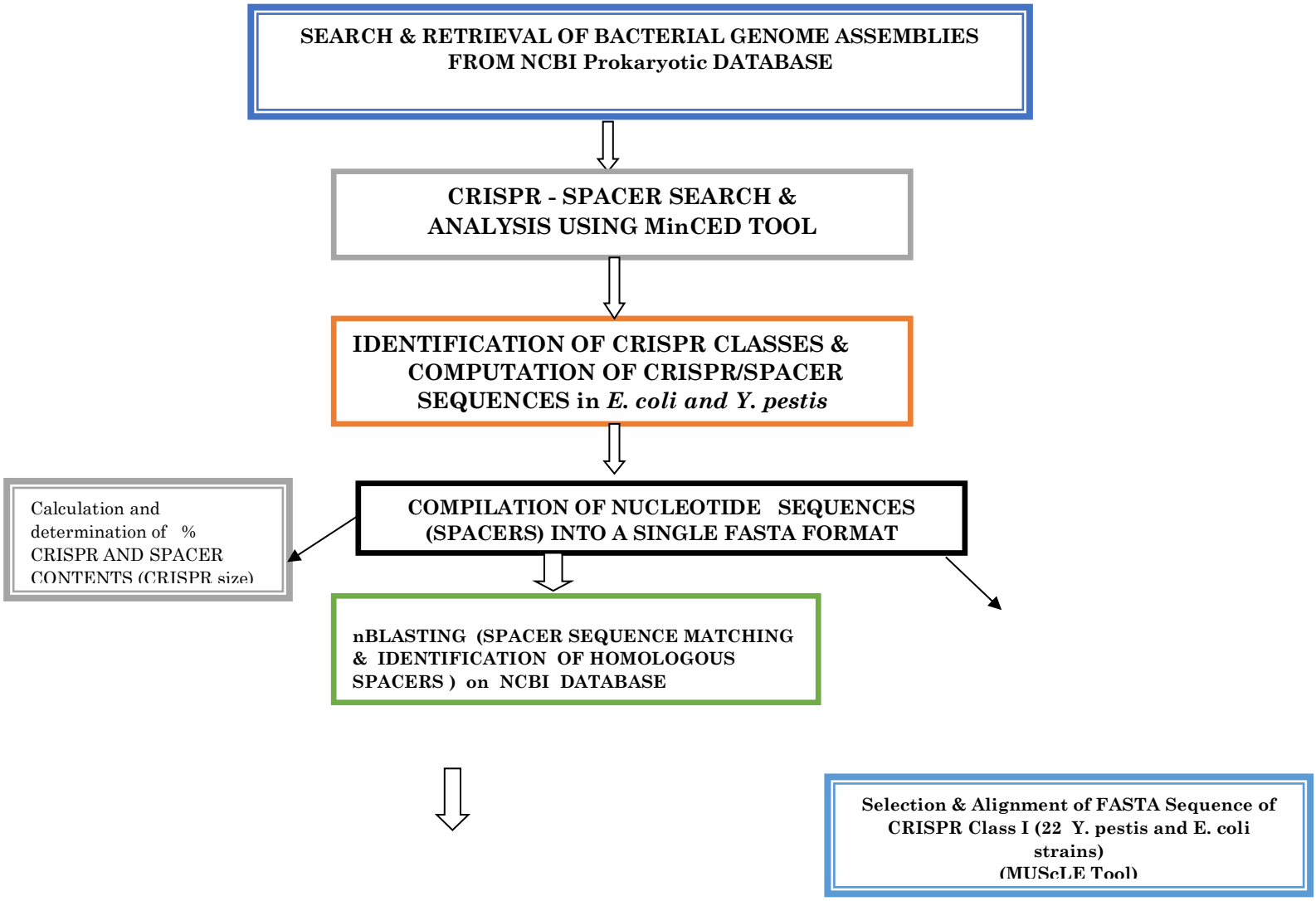
The "One Click mode" targets users that do not wish to deal with program and parameter selection. By default, the pipeline is already set up to run and connect programs recognized for their accuracy and speed (MUSCLE for multiple alignments and PhyML for phylogeny) to reconstruct a robust phylogenetic tree from a set of sequences.

In the "Advanced mode", the Phylogeny.fr server proposes the succession of the same programs, but users can choose the steps to perform (multiple sequence alignment, phylogenetic reconstruction, tree drawing) and the options of each program.

The "A la carte mode" offers the possibility of running and testing more alignment and phylogeny programs: MUSCLE, ClustalW, T-Coffee, PhyML, BioNJ, TNT. Alternatively, users can run the different programs on its web interface separately.

2.2 Experimental Design and Flow Chart

The schematic presentation of the experimental procedures performed in this research study is summarized below (Figure 2.2);



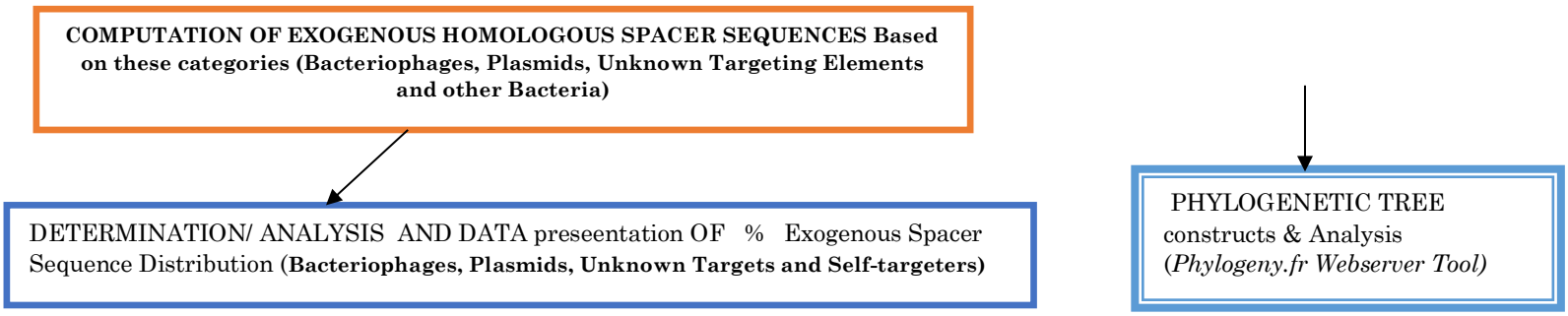


Figure 2.2: Schematic diagram of flow chart

Procedure:

i. Search and download of meta-genomic sequences from NCBI database and MinCED analysis:

A total of 258 complete meta-genome sequences of *E. coli strains* (120,000–5,000,000bp) were retrieved and downloaded from the NCBI database. The MinCED tool was used to analyze CRISPR-spacer content of 258 genome assemblies and **162** *E. coli* strains showed were identified with CRISPR and spacers sequences, while 96 *E. coli* strains showed absence of CRISPRs-spacers.

A total of 250 complete meta-genome sequences of *Yersinia pestis* strains (100,000 – 4,000,000 bp) were retrieved and downloaded from the NCBI prokaryotic database. The CRISPR analysis of 250 *Yersinia pestis* strains with the aid of the MinCED tool showed that only **121** *Y. pestis* strains had CRISPR and spacers, while 129 strains showed absence of CRISPR-spacers.

ii. nBLAST of spacer sequences (Homologous spacer search)

E. coli:

A total of **2,767** spacers earlier identified in 4 CRISPR classes of 162 Escherichia coli strains were subjected to a nucleotide mega BLAST on the NCBI database. The result of homologous exogenous spacers match (100% alignment score and e-value 30-60) was obtained from the NCBI database

A total of **9,605** hit spacer sequences were obtained from the NCBI database, after subjecting 2767 spacers to the nucleotide mega BLAST. A total number of 9,605 hit spacer sequences of bacteriophages, plasmids, bacterial strains, and unknown targets identified from NCBI database showed exact homologous sequence matches with 2,767 spacers derived from 4 E. coli CRISPR classes.

Y. pestis:

A total of **1,500** spacers earlier identified in 3 CRISPR classes of 121 Yersinia *pestis* strains were subjected to a nucleotide mega BLAST on the NCBI database. The result of homologous exogenous spacers match (100% alignment score and e-value 30-60) was obtained from the NCBI data bank. A total of **4,792** hit spacer sequences were obtained from the NCBI database, after subjecting 1,500 spacers to a nucleotide mega BLAST.

A total number of **4,792** spacers' sequences of bacteriophages, plasmids, bacterial strains, and unknown targets identified from NCBI database showed exact homologous sequence matches with 2,767 spacers derived from 4 E. coli CRISPR classes.

Data presentation: Statistical Multiple Bar charts and tables were used to present and analyze CRISPR and spacer sizes of the two bacterial species, percentage distribution of CRISPR-spacers amongst CRISPR classes of *Escherichia coli* and *Yersinia pestis* strains.

iii. Procedure for generating Phylogenetic tree constructs:

The representative members of *E. coli* and *Yersinia pestis* strains were separately analyzed using phylogenetic trees, to determine any possible evolutionary relationships associated with the CRISPR-spacers of selected representatives of 162 *Escherichia coli* and 121 *Yersinia pestis*. The selection was done to determine temporary relationship within the strains over a specified period [n<10 years]. This was achieved through the selection of spacers' sequences of CRISPR class I and aligning them into a single FASTA or PHYL or Nerwick format using MuSCLE bioinformatics tool.

Spacer sequences of CRISPR class I belonging to 22 representatives of E. coli strains were separately collected and aligned into a single FASTA file format, with the aid of the MUSCLE alignment program tool, available and accessible on the webpage (http://www. phylogeny.fr). The phylogenetic trees (dendograms) were constructed by inputting the single nucleotide FASTA formats into web server (http://www.phylogeny.fr).

Similarly, spacers' sequences of CRISPR class I belonging exclusively to 22 representatives of Y. pestis was selected and aligned into a single FASTA file, with the aid of the MUScLE Alignment program tool available on the *Phylogeny* web interface, (http://www. phylogeny.fr). The phylogenetic tree was constructed by inputting the single FASTA formatted file in the database.

E. coli and Y. pestis: The spacers' sequences of CRISPR class I comprise 3 *Y. pestis* and 3 *E. coli* strains were selected, to determine the extent of evolutionary relationships between the CRISPR class I spacers of *Yersinia pestis* and *E. coli.* The aligned FASTA nucleotide spacer sequences of CRISPR class I were analyzed using *Phylogeny.fr,* an online web-based phylogenetic analytical tool. Strains that had a closest phylogenetic relationship were identified and established, based on sister taxa / or a group of strains (with most recent root and branch) showing a common ancestral origin.

RESULTS AND DISCUSSION

3.0 RESULTS

3.1 PRELIMINARY ANALYSIS OF E. coli META-GENOMIC SEQUENCES

. Table 3.1 showed the CRISPR size, Spacer compositions of *Escherichia coli and* 4 CRISPR classes (CRISPR I, II, III, and IV) were present, and randomly distributed in 162 Escherichia coli strains.

Table 3.1: CRISPR and Spacers compositions of *Escherichia Coli*

CRISPR Class	Number of CRISPR	N umber of Spacers
CRISPR class I	162	1,512
CRISPR class II	132	1,167
CRISPR class III	15	79
CRISPR class IV	3	9
TOTAL	**312**	**2,767**

From Table 3.1, the result values obtained have shown that 162 E coli strains had a CRISPR and spacer size comprising 312 CRISPR classes and 2,767 spacers accordingly.

A total number of 312 CRISPR sequences were found in E. coli and distributed unevenly in 4 CRISPR classes of E. coli strains as follows: **Class I (162), Class II (132), Class III (15), and Class IV (3).**

The total number of 2,767 spacers sequences were associated with 4 CRISPR classes of E. coli and distributed as follows: **CRISPR class I** (1,512 spacers), **CRISPR Class II** (1,167 spacers), **CRISPR class III** (79 spacers), **and CRISPR class IV** (9 spacers).

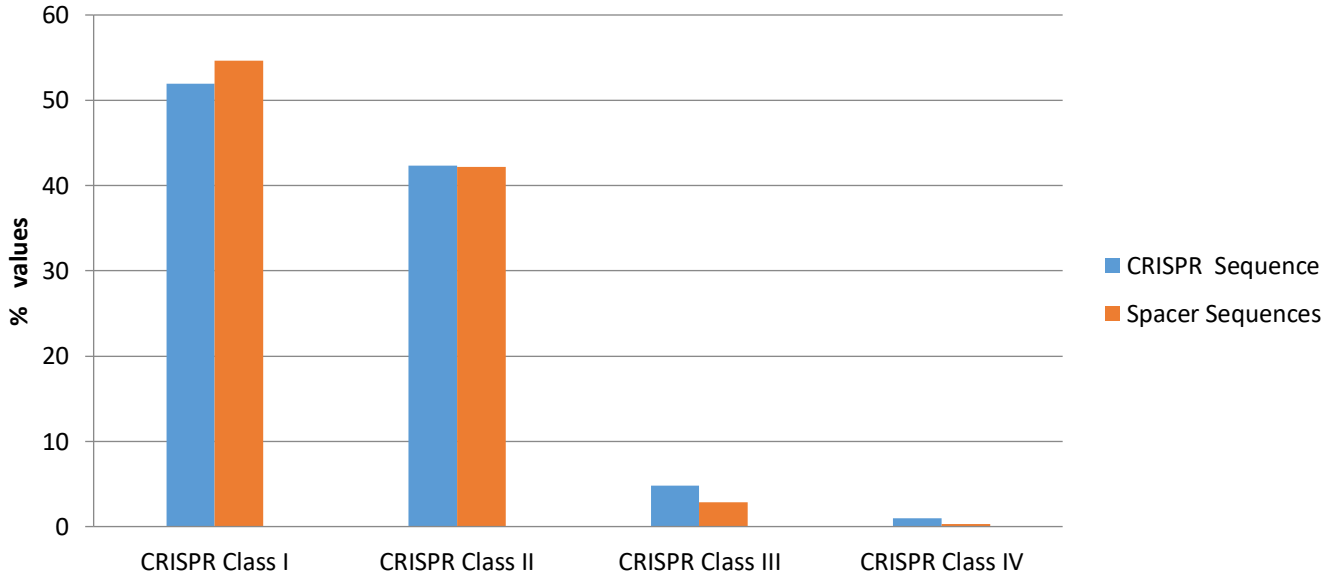


Figure 3.1: Percentage CRISPR and spacers compositions of E. *coli.*

The percentage distribution chart of CRISPR size and spacer content of E. coli as shown in Figure 3.1 illustrates the distribution of percentage values of CRISPR and spacer sequences among CRISPR Classes identified in 162 E. coli strains.

The result indicated that the percentage distribution values of CRISPR and spacers among 4 CRISPR classes of E. coli showed that the CRISPR class I had the highest percentage value of 54.64%, followed by 42.18% CRISPR II (42.18%), CRISPR III (2.86%) and CRISPR IV (0.33%). The least percentage values were recorded mainly by CRISPR class IV and CRISPR class III, respectively.

The significance of these preliminary findings explicitly indicated that 162 E. coli strains had 4 major CRISPR classes, with a high preponderance of CRISPRs present in class I and class II and thus suggests presence of CRISPR class I and its subtypes, and spacer content. This further explains the possibility of high CRISPR diversity in Class I and II compared with CRISPR class III and class IV.

3.1.2 PERCENTAGE DISTRIBUTION OF EXOGENOUS SPACERS in *E. coli* CRISPR classes

The result presented in Table 3.1.2 showed the distribution of homologous spacer sequences derived from Bacteriophages, Plasmids, Unknown targeting elements and other bacterial strains, respectively.

Table 3.1.2: Spacer distribution of Escherichia *coli CRISPR* classes.

CRISPR Class	Bacteriophages	Plasmids	Other Bacteria	Unknown targets
CLASS I	**53**	**5,448**	**671**	**22**
CLASS II	**49**	**2,947**	**294**	**87**
CLASS III	**0**	**16**	**18**	**0**
CLASS IV	**0**	**0**	**0**	**0**
TOTAL	**102**	**8,411**	**983**	**109**

Table 3.1.2 above showed the exact values of homologous spacers matches of E. coli CRISPR compared to other exogenous spacers. It further showed that the total number of 102 bacteriophages spacers, 8,411 plasmid spacers, 109 spacers from unknown targets, and 983 spacers identified from bacterial strains were found in the NCBI databank.

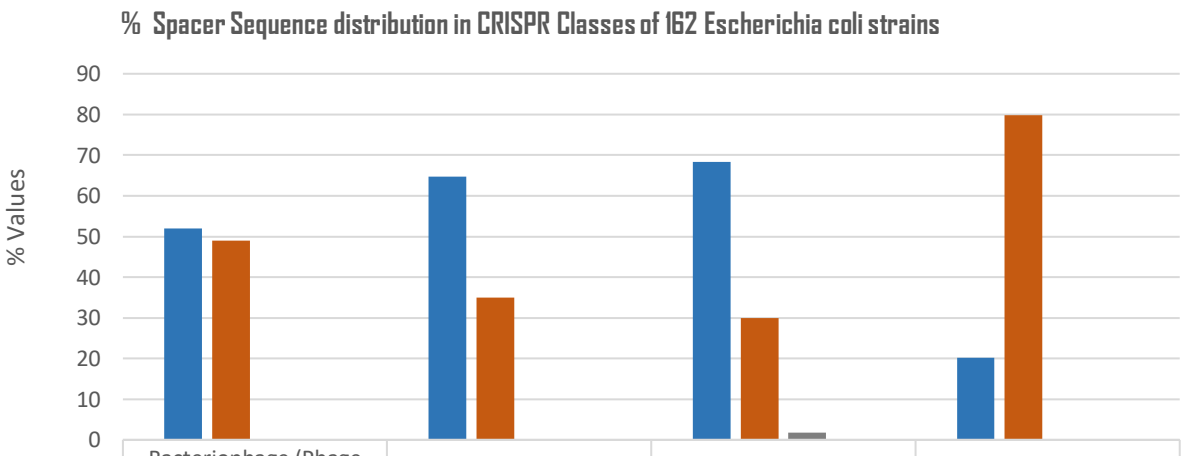
Results interpretation:

The result indicated that the CRISPR class I had a total number of **6,194 spacers**, with significant portions of homologous spacers matches from bacteriophages (53 spacers), plasmids (5,448 spacers), other bacterial organisms (671 spacers), and unknown targets (22 spacers) accordingly.

The **CRISPR class II** had a total number of **3,377 spacers**, with significant distributions from bacteriophages (49 spacers), plasmids (2,947 spacers), other bacterial organisms (294 spacers), and unknown targets (87 spacers).

The result further indicated that the **CRISPR class III** had the total number of **34 spacers** which showed resemblance with plasmids (16 spacers) and other bacterial strains (18 spacers). However, no homologous spacer sequence match with these exogenous elements was identified in CRISPR class IV.

Figure 3.1.2 below showed the summary of the percentage distribution chart of exogenous spacers sequence match between the spacer sequences of E. coli strains and exogenous elements (bacteriophages, plasmids, unknown targets, and other bacterial strains).



	Bacteriophage (Phage Virus)	Plasmids	Other Bacteria	Unknown targets
Class I	51.96	64.77	68.26	20.18
Class II	49	35	29.9	79.82
Class III	0	0.19	1.83	0
Class IV	0	0	0	0

Figure 3.1.2: Percentage Spacer distribution of E coli CRISPR classes

Results Interpretation:

i) *% Bacteriophages*: It further revealed that the *E. coli* **CRISPR I** had the highest percentage value of 52% homologous sequence matches with spacers from bacteriophages, and the E.

coli CRISPR class II recorded 49% homologous spacers matches. However, it was observed that no homologous spacer matches were recorded in the E. coli CRISPR class III and IV respectively.

ii) *% Plasmid:* From the above distribution chart, it showed that **CRISPR class I** showed the highest percentage value of 68.26% homologous sequence matches with plasmids spacers while Class II had 35% , CRISPR class III (0.19%) and CRISPR class IV showed no homologous sequence match with plasmids' spacers available in the NCBI repositories.

iii) % *Exogenous bacteria:* The result indicated that *E. coli* **CRISPR Class I** showed a significant percentage proportion (68.26%) of homologous sequence matches with exogenous bacterial spacers, while CRISPR class II and CRISPR class III showed 29.9% and 1.83% homologous sequence matches with exogenous bacterial spacers from NCBI repository. It was also observed that CRISPR class IV showed no percentage homologous sequence matches with spacers from exogenous bacteria.

iv) % *Unknown targets: E. coli* **CRISPR class II** showed 79.8% homologous sequence matches with spacers from unknown targeting elements, while CRISPR class I showed 20% homologous sequence matches with spacers from unknown targets. It was further observed that there were no homologous sequence matches recorded by E. coli CRISPR class III and IV with spacers from unknown targets.

The significance of this result showed that a significant variation in the patterns of spacer distribution and acquisitions by the E. coli CRISPR Classes. However, it was observed that E. coli CRISPR class I showed highest percentage values of homologous sequence matches with spacers from bacteria, bacteriophages and plasmids, and lesser percentage homologous spacers from unknown targets. In addition, the pattern of the percentage spacer distribution of exogenous spacers in the CRISPR class II varied significantly. However, CRISPR class II significant percentage proportions of homologous spacers were derived from unknown targets.

It was observed that there was a non-significant percentage proportions of homologous spacers sequence match found in the CRISPR class III as compared with CRISPR class I and class II.

However, the non-significant proportions of homologous spacers sequence matches found in CRISPR class III were mainly derived from plasmid and exogenous bacterial plasmids. The CRISPR class IV showed no homologous sequence match with exogenous spacers available in the NCBI repositories.

The implication of these findings thus further suggests that the E. coli bacterial strains could have acquired significant portions of their spacers' sequences from these exogenous elements, with highest exogenous spacers acquired by CRISPR class I, followed by CRISPR class II and CRISPR class III. It was observed that CRISPR diversity of E. coli strain could have been contributed by significant proportion of exogenous spacers acquired from homologous spacers often associated with plasmid, bacteria, unknown targets, and bacteriophages. The CRISPR diversity of E. coli might have been significantly due to incessant exposure of numerous E. coli bacterial strains, to infectious attack by bacteriophages, conjugation, and co-evolution of E coli strains with other exogenous elements in the environment.

3.2 PRELIMINARY ANALYSIS OF *Yersinia pestis* METAGENOMIC SEQUENCES

Table 3.2 showed the CRISPR and Spacer compositions of *Yersinia pestis*. The result showed that 3 CRISPR classes namely CRISPR I, II and III were present while CRISPR-spacer of Class IV was not found in 121 *Yersinia pestis* strains. The preliminary result of the meta-genomic CRISPR analysis of 121 *Yersinia pestis* were tabulated as shown in Table 3.2

Table 3.2: CRISPR and spacer compositions of *Yersinia pestis* classes

CRISPR classes	Number of CRISPR	Number of Spacers
CRISPR class I	121	554
CRISPR class II	110	617
CRISPR class III	71	329
CRISPR class IV	0	0
Total	302	1,500

From Table 3.2, the result values of 121 Yersinia pestis strains showed 302 CRISPR size and 1,500 spacer contents accordingly.

A total number of 302 CRISPR sequences were distributed in 3 CRISPR classes of Y. pestis strains as follows: **Class I (121), Class II (110), Class III (71), and Class IV (0).**

Total numbers of 2,767 spacers' sequences were associated with 3 CRISPR classes of *Yersinia pestis*, and their distributions are as follows: **CRISPR class II** (617 spacers), **CRISPR Class I** (554 spacers) **and CRISPR class III** (329 spacers). It is showed that there was absent of CRISPR class IV which further suggests absence of CRISPR and spacer sequences.

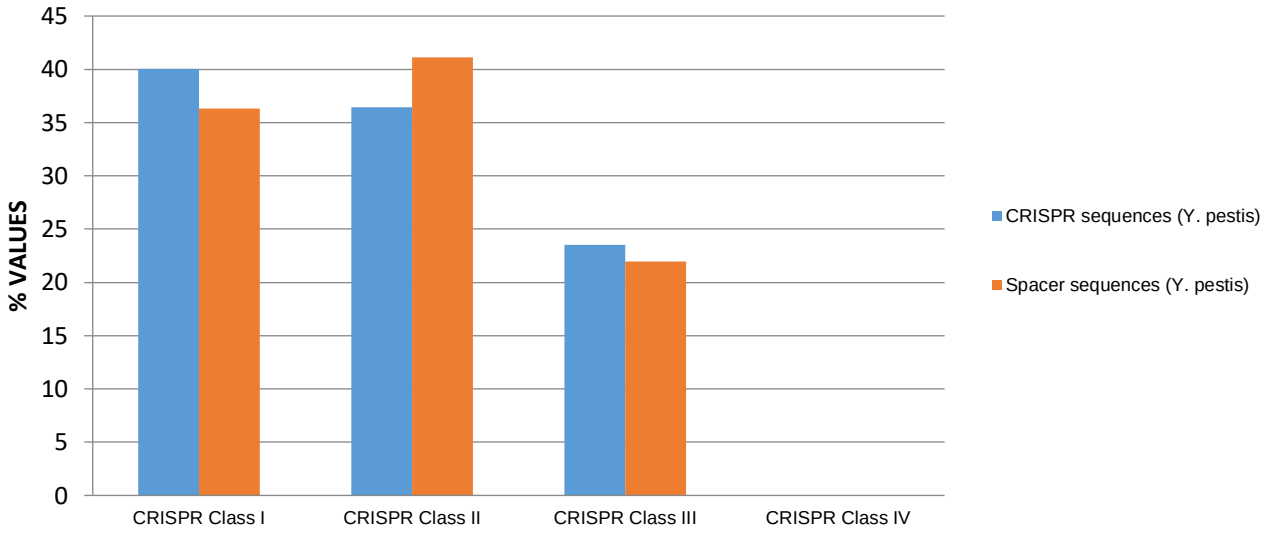


Figure 3.2: Percentage CRISPR and spacers compositions of Yersinia *pestis* classes

Figure 3.2 showed the percentage distribution chart of CRISPR size and spacer content of *Y. pestis*. The result indicated that the percentage distribution values of CRISPR and spacers in 3 CRISPR classes of Yersinia pestis showed that the CRISPR class I had the highest percentage value of 40%, followed by CRISPR class II (36.9%), CRISPR class III (21.93%) and CRISPR class IV (0%). The least percentage values were recorded mainly by CRISPR class IV and CRISPR class III, respectively.

The significance of these findings further shown that a high percentage proportions of CRISPR sequences composition was found in CRISPR class I than CRISPR classes II and III respectively. It further indicated that the CRISPR class I had the least CRISPR size than CRISPR class I and class II, while CRISPR class III. The findings from the above results have remarkably showed a significant difference in CRISPR sizes, regarding the number of CRISPR classes, CRISPR sequences and spacer contents compared with E. coli CRISPR-cas system.

3.2 PERCENTAGE DISTRIBUTION OF EXOGENOUS SPACERS in *Yersinia pestis* CRISPR classes.

Table 3.2.1 showed the exact distribution of homologous spacer sequences from Bacteriophages, Plasmids, Unknown targeting elements and other bacterial strains, respectively. The results of homologous spacers sequences were identified from bacteriophages, plasmids, bacterial strains, and unknown targets. The preliminary data were computed and tabulated as shown in Table 3.2.1 below.

Table 3.2.1: Spacer distribution of *Yersinia pestis* CRISPR classes

CRISPR Class	Bacteriophages	Plasmids	Other Bacteria	Unknown targets
CLASS I	50	149	1654	0
CLASS II	27	98	1699	0
CLASS III	28	138	949	0
CLASS IV	0	0	0	0
TOTAL	105	385	4,302	0

Table 3.2.1 above showed the exact values of homologous spacers matches between *Y. pestis* spacers and other spacers from exogenous elements. It further indicated that a total number of 105 bacteriophages spacers, 385 plasmid spacers and 4,302 bacterial spacers, while no homologous spacer sequence matches were found with any unknown targets.

Results interpretation:

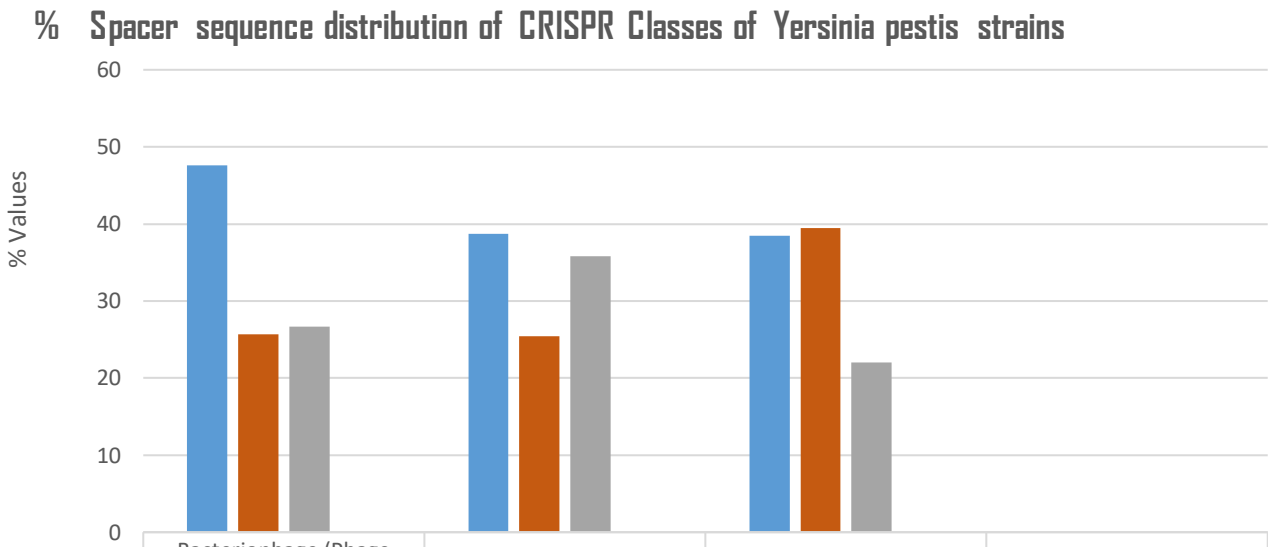
The result indicated that the **CRISPR class I** had a total number of **1,853 spacers**, with significant portions of homologous spacers matches identified from bacteriophages (50 spacers), plasmids (149 spacers), other bacterial organisms (1,654 spacers), and no spacer sequence were identified with unknown targets accordingly.

The **CRISPR class II** had a total number of **1,824 spacers**, with significant distributions from bacteriophages (27 spacers), plasmids (98 spacers), other bacterial organisms (1699 spacers), and no spacers from unknown targets.

CRISPR class III had a total number of **1,115 spacers**, with some portions of its homologous spacers from plasmids (138 spacers), bacteriophages (28 spacers) and other bacterial strains (949

spacers). However, no homologous spacer sequence match with any unknown elements was identified in CRISPR class IV.

Figure 3.2.1 below showed the summary of the percentage distribution chart of exogenous spacers sequence match between the spacer sequences of Y. pestis strains and exogenous elements (bacteriophages, plasmids, unknown targets, and other bacterial strains).



	Bacteriophage (Phage Virus)	Plasmids	Other Bacteria	Unknown targets
Class I	47.62	38.7	38.44	0
Class II	25.71	25.45	39.49	0
Class III	26.67	35.84	22.06	0
Class IV	0	0	0	0

Figure 3.2.1: Percentage spacer distribution of exogenous spacers in *Y. pestis* CRISPR classes

Results Interpretation:

i) *% Bacteriophages*: It further revealed that the **CRISPR I** had the highest percentage value (47.6%) of homologous sequence matches with spacers from bacteriophages, and the CRISPR class III (26.7%) and CRISPR class II (25.7%) homologous spacers matches. However, it was observed that no homologous spacer matches were recorded in the Yersinia pestis CRISPR class IV respectively.

ii) *% Plasmid:* From the above distribution chart, it showed that **CRISPR class I** showed the highest percentage value (38.7%) homologous sequence matches with plasmids spacers compared with the CRISPR Class III (35.8%) and CRISPR class II (25.5%). CRISPR class

IV showed no homologous sequence match with plasmids' spacers available in the NCBI repositories.

iii) % *Exogenous bacteria:* The result indicated that **CRISPR Class II** showed a significant percentage proportion (39.49%) of homologous sequence matches with exogenous bacterial spacers, while CRISPR class I and CRISPR class III showed 38% and 22% homologous sequence matches with exogenous bacterial spacers from NCBI repository.

iv) % *Unknown targets:* there was no homologous spacer sequence match found in any of the *Y. pestis* CRISPR classes.

The significance of this result showed significant variations in the pattern of spacer distribution and acquisitions by Y. pestis CRISPR Classes. However, it was observed that Y. pestis CRISPR class I showed highest percentage values of homologous sequence matches with spacers from bacteria, bacteriophages, and plasmids. In addition, the pattern of the percentage spacer distribution of exogenous spacers in the CRISPR class II varied significantly. However, CRISPR class II contributed significant percentage proportions of homologous spacers derived mainly from exogenous bacteria.

It was observed that CRISPR class III spacers derived some proportions of its spacers from plasmids, bacteriophages, and other bacterial plasmids, with absence of homologous spacers from unknown targets.

The finding suggests that Y. pestis bacterial strains might have acquired significant portions of their spacers' sequences from these exogenous elements excluding unknown targets. The significant proportion of spacers acquired by the bacteria was contributed by CRISPR class I, and CRISPR class II, while CRISPR class III had a least percentage spacer distribution. A comparison of the CRISPR size and spacer content of Y, pestis have clearly showed that Y. pestis CRISPR diversity is lesser compared with E. coli CRISPR systems, in terms of its CRISPR size, spacer sequence content and CRISPR class. The spacer distributions of E. coli strains were widely contributed by exogenous plasmids, bacteriophages, bacteria, and unknown target, whereas the percentage proportions of spacers acquired by Y. pestis CRISPRs are mainly derived from plasmids, bacteriophages and bacterial spacers only. This further implied that there is tendency of increased CRISPR diversity in E. coli strains than Y. pestis, despite the fact that both bacterial species are constantly exposed to infectious attack from bacteriophages and conjugation, and co-evolution of E coli strains from other exogenous elements in the environment.

3.3 PHYLOGENETIC STUDIES OF *Escherichia coli* and *Yersinia pestis* strains

3.3.1 Phylogenetic analysis of *Escherichia coli* strains

The phylogenetic tree showing the evolutionary relationships of 22 representative strains of Escherichia *coli* as obtained from the *phylogeny, a* web-server bioinformatics tool, and the result is presented (Figure 3.3.1) below;

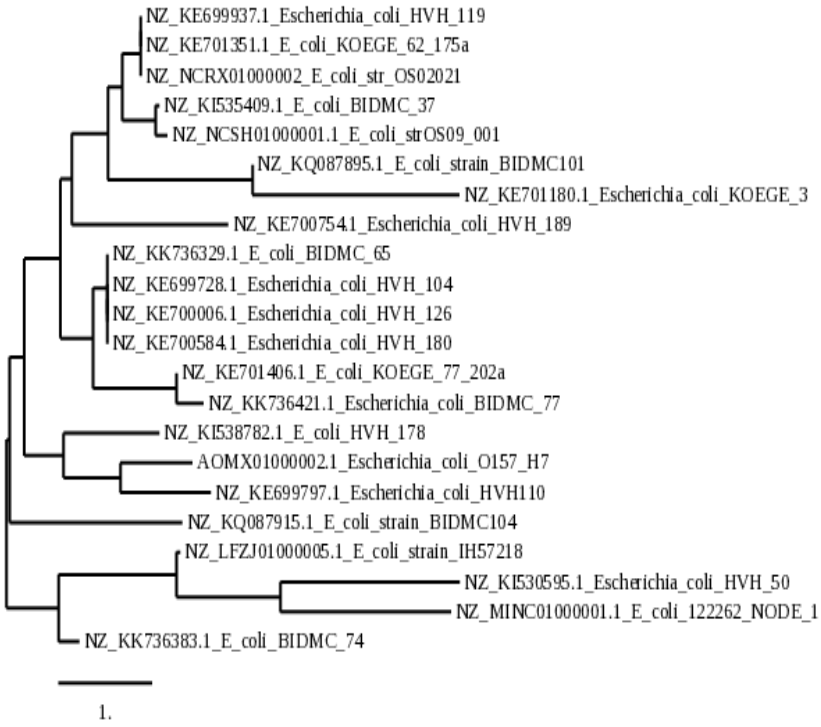


Figure 3.3.1: Phylogenetic tree of *E. coli strains*

From figure 3.3.1, the result showed the phylogenetic relationship of 22 representatives of 162 E coli strains. The analysis of the result of the dendrogram clearly showed that 22 representative strains had a common ancestral origin from E coli BIDMC 74(*Accession id*: NZ_KK736383.1).

E. coli BIDMC_74 strain showed a distant relationship from three E coli bacterial strains (E. coli HVH str. 119, E. coli KOEGE 62[175a], and E coli strain OSO2021). In Figure 3.3.1, it further showed that 19 strains (out of 22 E. coli strains) had a common ancestral origin with E coli

BIDMC_74, despite showing a distant relationship with E. coli BIDMC_74 because of divergent roots and branches linking 19 strains together with their origin. E coli BIMC_74 strain is closely related to E coli strain IH57218 compared with two other bacterial strains (E coli str. HVH 50 and E. coli str. 122262 NODE_1). However, E. coli strain strain_H57218 was closely related with sister taxa (E. coli strains HVH_50 and E coli NODE_1).

It was equally found that a sister taxa comprising four closely related E. coli strains (E coli str. BIDMC_65, E. coli str. HVH 104, E..coli 126, and E. coli 180) and another sister taxa slightly related 2 E. coli strains (E coli str. KOEGE 77[202a] and E. coli str. BIDMC 77) showed a close evolutionary relationship due to presence of a common root linking them together in the phylogenetic tree. The phylogenetic tree showed presence of divergent branches and roots that separately linked some sister taxa (groups) of E. coli strains together, despite having a common ancestral origin.

3.3.2 Phylogenetic analysis of *Yersinia pestis* strains

The result showed that the phylogenetic tree of the 22 representative strains of this bacterial species were obtained, and was presented in figure 3.3.2 below.

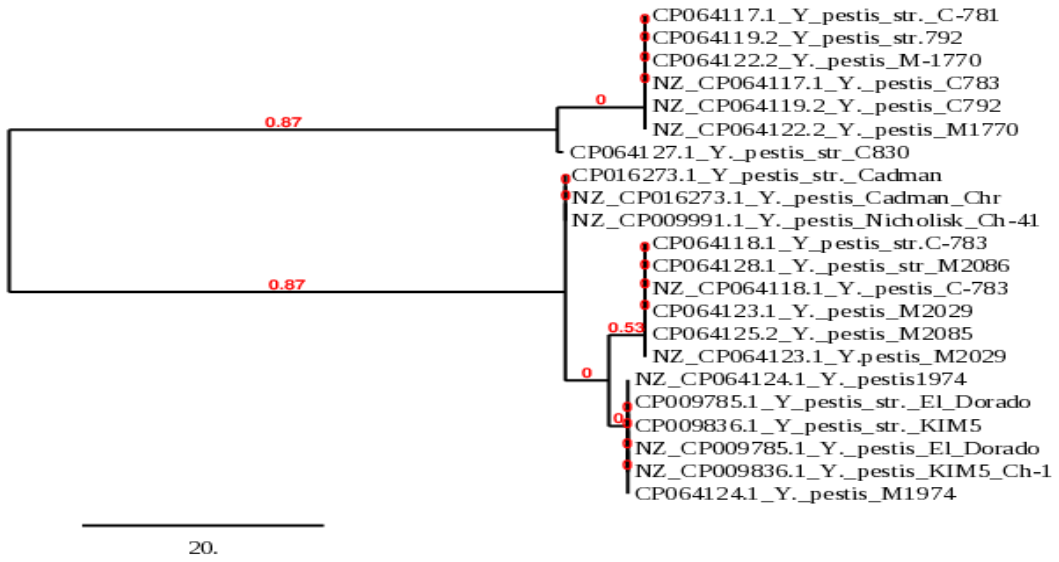


Figure 3.3.2: Phylogenetic tree of *Yersinia pestis* strains

Figure 3.3.2 showed the phylogenetic tree of 22 representatives of 121 Yersinia pestis strains, which were compared together, to elucidate their evolutionary relationships of *Y. pestis* strains.

The result showed that the 22 Yersinia pestis strains were closely related and could have originated from any of these Y. pestis strains [Y. pestis str. C830, Y. pestis str. Cadman, Y. pestis str. sub species Cadman Chr_1 and Y. pestis str. Chr_41] showed in the phylogenetic tree.

It was observed that 22 representatives were more closely related, because they had a common root of origin. Thus, the close relationship existed among different strains belonging to each group as categorized separately: **group A** (Yersinia pestis str. C.78, Y. pestis str. 792, Y. pestis str. M1770, Y. pestis str. C.792, Y. pestis str. C. 783 and Y. pestis str.M.170); **group B** (Y. pestis Cadman, Y. pestis Cadman Chr_1 and Y. pestis Nicholisk chr_41, **group C** (Y. pestis str. C.783, Y. pestis str. M2086, Y. pestis str. subsp. C.783, Y. pestis str. M2085 and Y. pestis str. M2029), **group D** (Y. pestis str. 1974, Y. pestis str. El Dorado, Y. pestis str. KIM5, Y. pestis KIM5 subsp. Chr 1 and Y. pestis str. M1974).

It is shown that there is a slight evolutionary relationship between *Y. pestis* strains belonging to group C and group D with a phylogenetic distance {d= 0.53}, while groups: A, B and D are more closely related respectively.
Since the 22 representative strains showed a close relationship with one another as compared to E. coli strains, it was observed that there were few branches, except groups C and D though both groups shared same root of origin. It can further be elucidated that spacer sequences of CRISPR class I genes of *Y. pestis* strains showed a significant close relationship with one another than E. coli strains. Thus, it suggests that there is little or no significant CRISPR diversity in CRISPR class I spacers of 22 Yersinia pestis strains which differ from E. coli strains.

3.3.3 Comparative phylogenetic analysis of *Yersinia pestis* and *Escherichia coli* species.

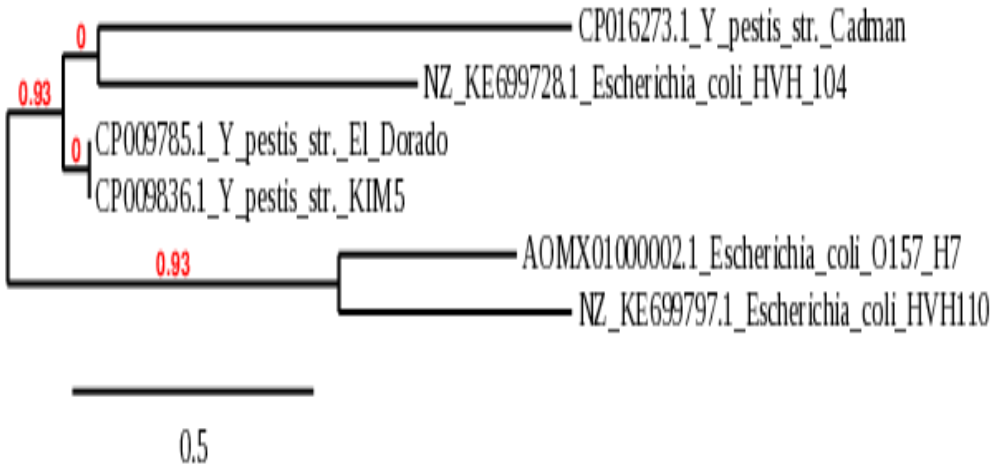


Figure: 3.3.3: Phylogenetic tree of *Yersinia pestis* and *E. coli* strains

The result of the comparative phylogenetic analysis of six bacterial strains comprising of 3 *Yersinia pestis* and 3 *E coli* as shown above in the Figure 3.3.3. There was a close relationship existing separately within the three sister taxa (strains belonging to each group), comprising of **Group A** {Y. pestis str. Cadman, E. coli str. HVH_104}, **Group B** {Y. pestis str. Eldorado and Y. pestis str. KIM5} and **Group C** {E. coli str. 0157H7 and E. coli HVH10}. The result of the phylogenetic tree (as shown in Figure 3.3.3) indicated a close relationship existing among the groups: A, B and C, which include these bacterial strains {Y. pestis str. Cadman [CP016273.1], Y. pestis str. El Dorado [CP009785.1], Y. pestis str. KIM5 [CP009836.1], Escherichia coli str. HVH 104 [NZ_KE699728.1], E. coli 0157_H7 [AOMX01000002.1] and E. coli str. HVH110 [NZ_KE699797.1]} because they had same root of origin.

However, it is observed that Escherichia coli strain HVH_104 and Y. pestis strain Cadman (Group A) is more closely related to Group B { Y. pestis str. El_Dorado and Y. pestis str. KIM5} than Group C {E. coli str. 0157H7 and E. coli HVH10} respectively. Although, the result of this study revealed that all these bacterial strains shared close evolutionary origin, despite a slight phylogenetic distance between group A and group C.

3.4 DISCUSSION

The results findings from this research study have compared the CRISPR –CAS systems of *Yersinia pestis* and *Escherichia coli* strains. Significantly, the result of MinCED-CRISPR

analysis showed that E. coli strain had 4 CRISPR classes (I, II, III and IV), and subsequent analysis of its CRISPR size and spacer content of 162 E. coli nucleotide genomes further revealed that a total of 312 CRISPR and 2,767 spacer sequences were present in 162 *E. coli* meta-genomic data sequences, with distribution of CRISPR Class I (162 CRISPR sequences), Class II (132 sequences), Class III (15 sequences), while CRISPR Class IV had only 3 CRISPR sequences. The percentage CRISPR size and spacer content showed that the CRISPR class I had the highest percentage value of 54.64%, followed by 42.18% CRISPR II (42.18%), CRISPR III (2.86%) and CRISPR IV (0.33%). The least percentage values were recorded mainly by CRISPR class IV and CRISPR class III, respectively. The significance of these preliminary findings explicitly indicated that 162 E. coli strains had 4 major CRISPR classes, with a high percentage composition of CRISPR sequences present in class I and class II, which further suggests that the CRISPR diversity of E. coli is largely contributed by CRISPR class I and II and their spacer contents. This finding therefore suggests that the CRISPR diversity of E. coli is largely contributed by Class I and II, when compared with CRISPR class III and class IV.

Contrarily, the result of the MinCED CRISPR analysis that determined CRISPR size and spacer content of 121 meta-genomic sequences of *Y. pestis*, however, identified mainly 3 CRISPR classes comprising Class I, II and Class III. The result further indicated that a total number of 302 CRISPR sequences were distributed in 3 CRISPR classes of Y. pestis strains as follows: Class I (121), Class II (110), Class III (71), and Class IV (0). The percentage values of CRISPR and spacer sequence contents of Y. pestis strains also revealed that the CRISPR class II had the highest CRISPR size (41.13%), while CRISPR class I (36.9%), CRISPR class III (21.93%) and CRISPR class IV (0%). The least percentage value was recorded by CRISPR class III, while no CRISPR sequences and spacers were present in CRISPR class IV, respectively. The total number of 2,767 spacers' sequences associated with 3 CRISPR Classes of *Yersinia pestis*, and their distributions showed that the CRISPR class II had 617 spacers, CRISPR Class I (554 spacers) and CRISPR class III (329 spacers), whereas CRISPR class IV showed absence of CRISPR and spacer sequences. The significance of this findings showed that Y. pestis had a lesser CRISPR size and spacer sequence content compared with E. coli strains.

The results of percentage distribution of exogenous spacers from plasmids (protospacers), bacteriophages (phage virus), bacterial species, and non-prophagic chromosomal regions (non-

targets) found in *Yersinia pestis* and *Escherichia coli* strains. The result showed that E coli and Y pestis had a total number of 9,605 and 4,792 homologous spacers sequences from exogenous elements were found in the NCBI data bank, excluding other CRISPR spacers.

The overall percentage proportions of homologous spacers from exogenous elements, in relation to 162 E. coli strain indicated the percentage values of spacers sequences acquired from plasmids (87.56%), bacteriophages (1.06 %), bacterial species (10.2%), and unknown targets (1.13%) in all CRISPR classes identified. It further showed that the percentage distribution of these spacers among E. coli CRISPR classes revealed that a significant percentage proportion of bacteriophages (51.96%) was found in CRISPR class I, 49% (CRISPR class II) while CRISPR III and IV had no spacers from bacteriophages. The percentage distribution of plasmids spacers also showed that 64.77% (CRISPR class I), 35% (CRISPR class II), 0.19% (CRISPR class III) while CRISPR IV had no spacers from plasmids. The percentage distribution of bacterial spacers: 68.26% (CRISPR class I), 29.9% (CRISPR class II), 1.83% (CRISPR class III), and no spacers were found in CRISPR class IV. In addition, it was found that the percentage distribution of unknown targets' spacers showed that 79.82 % (CRISPR class II), 20.18% (CRISPR class I), and no spacers (CRISPR class III and IV).

However, the total percentage contribution of homologous spacers from exogenous elements, in relation to 121 Y. pestis spacers indicated that 2.19% spacers were acquired from bacteriophages, 8% from plasmids and 89.8% bacteria. However, it was observed that there was no spacer sequence contributed by unknown targeting elements. The percentage spacer distributions across the 3 CRISPR classes showed as following: *% Bacteriophages*: CRISPR I had the highest percentage value (47.6%) of homologous sequence matches with spacers from bacteriophages, CRISPR class III (26.7%) and CRISPR class II (25.7%). However, it was also observed that no homologous spacer matches were found in the E. coli CRISPR class III and IV respectively. *% Plasmid:* CRISPR class I showed the highest percentage value (38.7%) homologous sequence, while CRISPR Class III (35.8%) and CRISPR class II (25.5%). Similarly, CRISPR class IV showed no homologous sequence match with plasmids' spacers available in the NCBI repositories. The percentage spacer distribution of *Exogenous bacteria* indicated that the CRISPR Class II showed a significant percentage proportion (39.49%) of homologous sequence matches with exogenous bacterial spacers, while CRISPR class I and CRISPR class III showed 38% and 22% homologous

sequence matches with exogenous bacterial spacers from NCBI repository. Also, it was showed that unknown target elements showed no homologous spacer sequence match with all Y. pestis CRISPR classes.

The phylogenetic analysis of the dendogram clearly showed that 22 representative strains had a common ancestral origin from E coli BIDMC 74(*Accession id*: NZ_KK736383.1). The phylogenetic tree showed presence of divergent branches and roots that separately linked some sister taxa (groups) of E. coli strains together, despite having a common ancestral origin. This however showed that there is increasingly huge diversity in the evolutionary relationships of CRISPR spacers of 22 E coli strains.

Analysis of the phylogenetic tree of 22 representatives of 121 Yersinia pestis strains further elucidated that there was a close evolutionary relationship among strains, because each strain was more closely related to one another. However, it can be showed that all Y. pestis strains had a closer linkage with Y. pestis strains [Y. pestis str. C830, Y. pestis str. Cadman, Y. pestis str. sub species Cadman Chr_1 and Y. pestis str. Chr_41].

More so, it was observed that there was a slight evolutionary relationship between Y. pestis strains belonging to group C and group D with a phylogenetic distance {d= 0.53}, while groups: A, B and D are more closely related respectively. It showed that there is an evolutionary relationship between Y. pestis strains belonging to group A and group D, while group C differs slightly from group A and D respectively. Despite having the presence of common ancestral origin from Yersinia pestis strain Cadman, few branching and phylogenetic distance, groups C and D shared the same root of origin which further implies that CRISPR class I genes of Y. pestis strains from these two groups had a common ancestral origin**.** It indicates that there is no wide diversity in CRISPR spacers of 22 Yersinia pestis strains.

The comparative phylogenetic study of the combined strains showed a close relationship within each of the three sister taxa comprising of Group A {*Y. pestis* str. Cadman, E. coli str. HVH_104}, Group B {*Y. pestis* str. El_Dorado and *Y. pestis* str. KIM5} and Group C {*E. coli* str. 0157H7 and *E. coli* HVH10}. However, it is observed that *Escherichia coli* strain HVH_104 and *Y. pestis* strain Cadman (Group A) is more closely related to Group B {*Y. pestis* str. El_Dorado and *Y. pestis* str. KIM5} than Group C {*E. coli* str. 0157H7 and *E. coli* HVH10} respectively. Although, the result

obtained from this study further revealed that all the six bacterial strains shared a close evolutionary relationship, in spite of the slight phylogenetic distance {*d=0.93*} between group A and group C. The findings from the phylogenetic analyses revealed have shown that there is an existing common evolutionary relationship between the CRISPR-Cas of *Yersinia pestis* and *Escherichia coli* strains.

4.0 CONCLUSION

The comparison of overall spacer sequence distribution of the CRISPR classes of 162 E. coli strain and *121 Y. pestis strains* further showed that a significant percentage proportion of homologous spacers were significantly contributed by plasmids (87.56%) in E. coli than Y. pestis strains (8%). Contrarily, exogenous bacterial spacers also contributed largely to CRISPR diversity of Y. pestis (89%) than E. coli (10.2%). The percentage distribution of bacteriophages spacers in E. coli (2.19%) and Y. pestis (1.06%) is not significantly different between two bacterial species. It is equally observed that the unknown target elements contributed a little percentage proportion (1.1%) in E. coli and contributed no spacers in Y. pestis strains. The significance of these results showed that there is significant variations in the pattern of spacer distribution and acquisitions in both E. coli and Y. pestis CRISPR Classes. However, E. coli CRISPR showed a wide CRISPR diversity than Y. pestis, in terms of its CRISPR size, spacer contents and phylogenetic relationship. In conclusion, the findings from the phylogenetic studies have indicated that the CRISPR spacers of the two different bacterial species, Yersinia pestis and E coli strains are closely related to each other, and the significant difference associated with the evolutionary relationships of these bacterial strains may be due to the pattern of CRISPR spacer acquisition by each bacterial strain from exogenous mobile elements. The findings from this study suggest the spacer sequences are derived from DNA fragments of bacteriophages and other exogenous elements that had previously infected the prokaryote and could be used to detect and destroy DNA from similar bacteriophages during subsequent infections. The findings thus showed that the increased CRISPR diversity in E. coli strains could be associated with increased exposure of its strains to bacteriophages' attacks, conjugation and co-evolution with other mobile exogenous elements like plasmid vectors, unknown targets and numerous bacterial strains in the environment and their host organisms.

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