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Distribution of CRISPR in *Escherichia coli* Isolated from Bulk Tank Milk and Its Potential Relationship with Virulence

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Abstract: *Escherichia coli* is one of the most common causes of mastitis on dairy farms around the world, but its clinical severity is determined by a combination of virulence factors. Recently, clustered regularly interspaced short palindromic repeat (CRISPR) arrays have been reported as a novel typing method because of their usefulness in discriminating pathogenic bacterial isolates. Therefore, this study aimed to investigate the virulence potential of *E. coli* isolated from bulk tank milk, not from mastitis, and to analyze its pathogenic characterization using the CRISPR typing method. In total, 164 (89.6%) out of 183 isolates possessed one or more of 18 virulence genes, and the most prevalent virulence gene was *fimH* (80.9%), followed by *iss* (38.3%), *traT* (26.8%), *ompT* (25.7%), *afa/draBC* (24.0%), and *univcnf* (21.9%). Moreover, the phylogenetic group with the highest prevalence was B1 (64.0%), followed by A (20.1%), D (8.5%), and C (7.3%) (*p* < 0.05). Among the four CRISPR loci, only two, CRISPR 1 and CRISPR 2, were found. Interestingly, the distribution of CRISPR 1 was significantly higher in groups A and B1 compared to that of CRISPR 2 (*p* < 0.05), but there were no significant differences in groups C and D. The prevalence of CRISPR 1 by virulence gene ranged from 91.8% to 100%, whereas that of CRISPR 2 ranged from 57.5% to 93.9%. The distribution of CRISPR 1 was significantly higher in *fimH*, *ompT*, *afa/draBC*, and *univcnf* genes than that of CRISPR 2 (*p* < 0.05). The most prevalent *E. coli* sequence types (EST) among 26 ESTs was EST 22 (45.1%), followed by EST 4 (23.2%), EST 16 (20.1%), EST 25 (19.5%), and EST 24 (18.3%). Interestingly, four genes, *fimH*, *ompT*, *afa/draBC*, and *univcnf*, had a significantly higher prevalence in both EST 4 and EST 22 (*p* < 0.05). Among the seven protospacers derived from CRISPR 1, protospacer 163 had the highest prevalence (20.4%), and it only existed in EST 4 and EST 22. This study suggests that the CRISPR sequence-typing approach can help to clarify and trace virulence potential, even though the *E. coli* isolates were from normal bulk tank milk.

Keywords: *E. coli*; virulence gene; phylogeny; CRISPR array; bulk tank milk
1. Introduction

*Escherichia coli* is one of the most common Gram-negative bacteria residing in the intestines of animals in an anaerobic and facultative manner [1]; however, it is also one of the most common causes of mastitis on dairy farms [2]. Generally, *E. coli* mastitis results in a subclinical pathology caused by an environmental opportunistic infection. However, the presence of diverse virulence properties associated with extraintestinal pathogenesis, such as adhesins, toxins, capsule synthesis, siderophores, invasins, and serum survival, is reported to be crucial for the colonization of the mammary glands via increased bacterial survival and tissue damage [3,4]. In particular, Aslam et al. (2021) [5] reported that the presence of various virulence genes in extraintestinal pathogenic *E. coli* (ExPEC) contributed to the rise in mammary pathogenic *E. coli* (MPEC).

Moreover, the virulence potential necessary to cause an infection of the mammary glands is determined by a combination of factors, not the presence of a single factor [6]. Hence, phylogenetic analysis is important because it enriches the understanding of the classification, and determines the virulence of pathogenic *E. coli* [7,8]. *E. coli* is derived from different phylogenetic groups A, B1, B2, C, D, E, and F [9], and the majority of strains responsible for ExPEC, such as uropathogenic *E. coli* (UPEC), newborn meningitis-associated *E. coli*, and avian pathogenic *E. coli*, belong to groups B2 and D [10,11]. However, even though MPEC can cause infections outside of the gastrointestinal system, both MPEC and bovine commensals belong to phylogroups A and B1, because MPEC may be recruited from the normal intestinal commensal microbiota [12–14].

Clustered regularly interspaced short palindromic repeat (CRISPR) arrays are a bacterial adaptive immune system that neutralizes invading phages and plasmids by cutting foreign DNA at specific locations. It consists of various spacers, which are short sequences between each repeat [15–17]. A protospacer, which is a short external sequence at a specific location, is inserted as a spacer in the CRISPR loci of bacteria during an infection [18]. Recently, CRISPR arrays have been applied as a novel typing method for isolates because they are useful in discriminating the pathogenicity of *Salmonella* [19], *E. coli* [20–25], and *Pseudomonas aeruginosa* [26]. This study aimed to investigate the virulence potential of *E. coli* isolated from bulk tank milk, not from mastitis, and to analyze the pathogenic characterization of *E. coli* using the CRISPR typing method.

2. Materials and Methods

2.1. Bacterial Strains

Each 50 mL of bulk tank milk was aseptically collected, from 290 farms operated by three dairy companies, and tested in accordance with the standard microbiological protocols published by the Ministry of Food and Drug Safety (2018) [27]. Approximately 1 mL of each bulk tank milk sample was inoculated into 9 mL of modified *Escherichia coli* broth (Merck, Darmstadt, Germany), and these were incubated at 37 °C for 24 h. A loopful of enriched mEC was streaked onto MacConkey agar (BD Bioscience), and incubated at 37 °C for 24 h. Three typical colonies selected from each sample were confirmed by PCR, as described previously [28]. If isolates of the same origin showed the same antimicrobial susceptibility patterns, only one isolate was randomly chosen, and a total of 183 *E. coli* were included in this study.

2.2. Detection of Virulence Genes

A total of 33 virulence genes associated with ExPEC (afa/draBC, bmaE, cdt, cdtB, cnf1, cvaC, fimH, focG, fyuA, hlyA, ibeA, ireA, iroNe. coli, iss, iutA, kpsMT K1, kpsMT2, kpsMT3, kpsMT K5, nfaE, ompT, PAI, papAH, papC, papEF, papG allele 1, papG allele 2, papG allele 3, rfc, sfa/focDE, sfaS, traT, and univcnf) were screened by PCR, as described previously [29].
2.3. Phylogenetic Groups

Phylogenetic grouping was accomplished by a multiplex PCR-based method using chuA, yjaA, TSPE4.C2, arpA, and trpA genes, and the bacteria were assigned into groups A, B1, B2, C, D, E, F, and clade I, as described previously [9].

2.4. CRISPR Locus Sequence Typing and Spacer Analysis

Four CRISPR loci were screened by PCR, as described previously [22]. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced using an automatic sequencer (Cosmogenetech, Seoul, Korea). Sequences were analyzed by CRISPRFinder (https://crispr.i2bc.paris-saclay.fr/Server/, accessed on 31 August 2021), as described by Grissa et al. (2007) [30], and only spacers were obtained for this study. All E. coli were categorized into E. coli sequence types (ESTs) based on their spacer distributions, numbered arbitrarily. The name and full sequences of all spacers are listed in Supplementary Table S1. CRISPRTarget (http://crispr.otago.ac.nz/CRISPRTarget/crispr_analysis.html, accessed on 31 August 2021) with a cut-off value of 29, and nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 10 September 2021) were used to detect protospacers derived from phages or plasmids [31].

2.5. Statistical Analysis

Analysis via the Statistical Package for the Social Sciences version 25 (IBM SPSS Statistics for Windows, Armonk, NY, USA) was used in this study. Pearson’s chi-square test and Fisher’s exact test with Bonferroni correction were conducted to analyze the differences associated with the distribution of virulence genes, phylogenetic groups, and ESTs. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Distribution of Virulence Genes

The distribution of 33 virulence genes associated with ExPEC in E. coli from bulk tank milk is presented in Table 1. A total of 164 (89.6%) out of 183 E. coli isolated from bulk tank milk carried one or more of eighteen virulence genes. The most prevalent virulence gene was fimH (80.9%), followed by iss (38.3%), traT (26.8%), ompT (25.7%), afa/draBC (24.0%), and univcnf (21.9%). Interestingly, both iss and traT had the significantly highest prevalence in E. coli from company A, and fimH had a significantly higher prevalence in E. coli from companies A and B (p < 0.05). Although kpsMTK5 had a low prevalence (7.1%) in E. coli, this gene also showed a significant difference among the companies (p < 0.05).

3.2. Distribution of Phylogenetic Groups and CRISPR Loci

The distribution of phylogenetic groups and CRISPR loci in 164 E. coli isolates with some virulence genes is presented in Table 2. All isolates were assigned into four phylogenetic groups: A, B1, C, and D. The phylogenetic group with the significantly highest prevalence was B1 (64.0%), followed by A (20.1%), D (8.5%), and C (7.3%). Although the distribution of groups B1 and C was not significantly different by company, that of groups A and D showed significant differences between companies (p < 0.05). Among the four CRISPR loci examined, only two, CRISPR 1 and CRISPR 2, were found. However, the prevalence of CRISPR 1 (95.7%) was significantly higher than that of CRISPR 2 (74.4%) (p < 0.05). On the other hand, no significant differences between the companies were observed.
Table 1. Distribution of virulence genes in E. coli isolated from bulk tank milk.

| Virulence Genes \(^1\) | No (%) of Isolates Included by Company |
|------------------------|---------------------------------------|
|                        | A \((n = 38)\) | B \((n = 42)\) | C \((n = 103)\) | Total \((n = 183)\) |
| fimH                   | 36 (94.7) \(a\) | 41 (100.0) \(a\) | 71 (69.0) \(b\) | 148 (80.9) \(A\) |
| iss                    | 27 (71.1) \(a\) | 14 (34.1) \(b\) | 29 (28.2) \(b\) | 70 (38.3) \(B\) |
| traT                   | 21 (55.3) \(a\) | 12 (29.3) \(b\) | 16 (15.5) \(b\) | 49 (26.8) \(BC\) |
| ompT                   | 10 (26.3)       | 14 (34.1)       | 23 (22.3)       | 47 (25.7) \(BC\) |
| afa/draBC              | 6 (15.8)        | 11 (26.8)       | 27 (26.2)       | 44 (24.0) \(BC\) |
| univcnf                | 6 (15.8)        | 15 (36.6)       | 19 (18.4)       | 40 (21.9) \(BC\) |
| iroN E. coli           | 5 (13.2)        | 8 (19.5)        | 12 (11.7)       | 25 (13.7) \(C,D\) |
| kpsMT K5               | 2 (5.3) \(a,b\) | 7 (17.1) \(a\) | 4 (3.9) \(b\) | 13 (7.1) \(D,E\) |
| fyuA                   | 5 (13.2)        | 3 (7.3)         | 4 (3.9)         | 12 (6.6) \(D,E\) |
| sfaS                   | 3 (7.9)         | 1 (2.4)         | 8 (7.8)         | 12 (6.6) \(D,E\) |
| bmaE                   | 0 (0.0)         | 0 (0.0)         | 9 (8.7)         | 9 (4.9) \(D,E\) |
| cnfI                   | 2 (5.3)         | 0 (0.0)         | 3 (2.9)         | 5 (2.7) \(E\) |
| cdt                    | 0 (0.0)         | 1 (2.4)         | 3 (2.9)         | 4 (2.2) \(E\) |
| hvjA                   | 1 (2.6)         | 1 (2.4)         | 1 (1.0)         | 3 (1.6) \(E\) |
| cdtB                   | 0 (0.0)         | 0 (0.0)         | 2 (1.9)         | 2 (1.1) \(E\) |
| iutA                   | 1 (2.6)         | 0 (0.0)         | 0 (0.0)         | 1 (0.5) \(E\) |
| papG allele 3          | 1 (2.6)         | 0 (0.0)         | 0 (0.0)         | 1 (0.5) \(E\) |
| kpsMT II               | 1 (2.6)         | 0 (0.0)         | 0 (0.0)         | 1 (0.5) \(E\) |

\(^1\) PAI, cvaC, focG, ibeA, ireA, kpsMT K1, kpsMT III, nfaE, papAH, papC, papEF, papG allele 1, papG allele 2, rfc, and sfa/focDE genes were not detected in any of the isolates. \(^2\) \(n\) = No. of E. coli isolated from each company. Values with different subscript letters represent significant differences among companies, while superscript letters represent significant differences in total \((p < 0.05)\).

Table 2. Distribution of phylogenetic groups and CRISPR loci of 164 E. coli possessing virulence genes isolated from bulk tank milk.

| Groups | No (%) of Isolates Included by Company |
|--------|---------------------------------------|
|        | A \((n = 38)\) | B \((n = 41)\) | C \((n = 85)\) | Total \((n = 164)\) |
| Phylogenetic groups | | | | |
| A       | 3 (7.9) \(b\) | 13 (31.7) \(a\) | 17 (20.0) \(ab\) | 33 (20.1) \(B\) |
| B1      | 26 (68.4) | 20 (48.8) | 59 (69.4) | 105 (64.0) \(A\) |
| B2      | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) \(D\) |
| C       | 1 (2.6) | 5 (12.2) | 6 (7.1) | 12 (7.3) \(C\) |
| D       | 8 (21.1) \(a\) | 3 (7.3) \(ab\) | 3 (3.5) \(b\) | 14 (8.5) \(C\) |
| E       | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) \(D\) |
| F       | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) \(D\) |
| CRISPR loci | | | | |
| CRISPR 1 | 37 (97.4) | 38 (92.7) | 82 (96.5) | 157 (95.7) \(A\) |
| CRISPR 2 | 33 (86.8) | 31 (75.6) | 58 (68.2) | 122 (74.4) \(B\) |
| CRISPR 3 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) \(C\) |
| CRISPR 4 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) \(C\) |

\(^1\) \(n\) = No. of E. coli isolated from each company. Values with different subscript letters represent significant differences among companies, while superscript letters represent significant differences in total \((p < 0.05)\).

3.3. Distribution of CRISPR 1 and CRISPR 2 by Phylogenetic Group

The association of CRISPR 1 and CRISPR 2 with phylogenetic groups of E. coli is shown in Figure 1. The prevalence of CRISPR 1 by phylogenetic groups ranged from 75.0% to 98.1%, whereas that of CRISPR 2 ranged from 60.6% to 92.9%. Interestingly, the distribution of CRISPR 1 and CRISPR 2 in the phylogenetic groups showed significant differences. The distribution of CRISPR 1 was significantly higher in groups A and B1 than that of CRISPR 2 \((p < 0.05)\). The prevalence of CRISPR 1 and CRISPR 2 showed no significant differences in groups C and D.
Figure 1. Distribution of CRISPR 1 and CRISPR 2 by phylogenetic group in 164 E. coli possessing virulence genes, isolated from bulk tank milk. The asterisk indicates that there were significant differences between CRISPR 1 and CRISPR 2 ($p < 0.05$).

3.4. Distribution of CRISPR 1 and CRISPR 2 by Virulence Genes

The association of CRISPR 1 and CRISPR 2 with six common virulence genes of E. coli is shown in Figure 2. The prevalence of CRISPR 1 by virulence gene ranged from 91.8% to 100%, whereas that of CRISPR 2 ranged from 57.5% to 93.9%. The distribution of CRISPR loci also showed differences by virulence gene. The distribution of CRISPR 1 was significantly higher in fimH, ompT, afa/draBC, and univcnf genes than that of CRISPR 2 ($p < 0.05$). Moreover, iss and traT genes showed equally high distributions, and no significant differences between CRISPR 1 and CRISPR 2.

Figure 2. Distribution of CRISPR1 and CRISPR2 by virulence gene in 164 E. coli possessing virulence genes, isolated from bulk tank milk. The asterisk indicates that there were significant differences between CRISPR 1 and CRISPR 2 ($p < 0.05$).

3.5. CRISPR-Based Typing of Virulence Gene-Carrying Isolates

The distribution of ESTs by six common virulence genes of E. coli is presented in Table 3. A total of 26 ESTs were assigned based on the distribution of spacers from CRISPR
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1 and CRISPR 2. The most prevalent EST was EST 22 (45.1%), followed by EST 4 (23.2%), EST 16 (20.1%), EST 25 (19.5%), and EST 24 (18.3%). Interestingly, four genes, *fimH*, *ompT*, *afa/draBC*, and *univcnf*, had a significantly higher prevalence in both EST 4 and EST 22, while *iss* and *traT* genes had a significantly lower prevalence than the other four genes in both EST 4 and EST 22 (p < 0.05).

Table 3. CRISPR-based typing by virulence gene in 164 *E. coli* possessing virulence genes, isolated from bulk tank milk.

| E. coli Sequence Types | *fimH* (n = 154) | *iss* (n = 70) | *traT* (n = 49) | *ompT* (n = 47) | *afa/draBC* (n = 44) | *univcnf* (n = 40) | Total (n = 164) |
|-----------------------|-----------------|---------------|----------------|-----------------|---------------------|------------------|---------------|
| EST 1                 | 0 (0.0)         | 2 (2.9)       | 0 (0.0)        | 5 (10.6)        | 3 (6.8)             | 9 (22.5)         | 19 (11.6)     |
| EST 2                 | 2 (1.4)         | 1 (1.4)       | 0 (0.0)        | 1 (2.1)         | 0 (0.0)             | 0 (0.0)          | 5 (3.0)       |
| EST 3                 | 3 (2.0)         | 2 (2.9)       | 2 (4.1)        | 1 (2.1)         | 0 (0.0)             | 0 (0.0)          | 8 (4.9)       |
| EST 4                 | 16 (10.8)       | 2 (2.9)       | 0 (0.0)        | 6 (12.9)        | 9 (20.5)            | 5 (12.5)         | 38 (23.2)     |
| EST 5                 | 3 (2.0)         | 1 (1.4)       | 0 (0.0)        | 1 (2.1)         | 0 (0.0)             | 0 (0.0)          | 6 (3.7)       |
| EST 6                 | 2 (1.4)         | 0 (0.0)       | 0 (0.0)        | 0 (0.0)         | 0 (0.0)             | 0 (0.0)          | 2 (1.2)       |
| EST 7                 | 2 (1.4)         | 0 (0.0)       | 1 (2.0)        | 0 (0.0)         | 1 (2.3)             | 0 (0.0)          | 4 (2.4)       |
| EST 8                 | 3 (2.0)         | 2 (2.9)       | 6 (12.9)       | 2 (4.3)         | 2 (4.5)             | 0 (0.0)          | 12 (7.3)      |
| EST 9                 | 3 (2.0)         | 1 (2.0)       | 1 (2.1)        | 1 (2.3)         | 0 (0.0)             | 0 (0.0)          | 8 (4.9)       |
| EST 10                | 7 (4.7)         | 4 (5.7)       | 2 (4.1)        | 3 (6.4)         | 0 (0.0)             | 2 (5.0)          | 18 (11.0)     |
| EST 11                | 3 (2.0)         | 3 (4.3)       | 2 (4.1)        | 0 (0.0)         | 0 (0.0)             | 0 (0.0)          | 4 (2.5)       |
| EST 12                | 1 (0.7)         | 0 (0.0)       | 1 (2.0)        | 0 (0.0)         | 1 (2.3)             | 0 (0.0)          | 3 (1.8)       |
| EST 13                | 7 (4.7)         | 5 (7.1)       | 1 (2.0)        | 1 (2.1)         | 2 (2.3)             | 0 (0.0)          | 15 (9.1)      |
| EST 14                | 8 (5.4)         | 6 (8.6)       | 8 (16.3)       | 0 (0.0)         | 2 (4.5)             | 1 (2.5)          | 25 (15.2)     |
| EST 15                | 2 (1.4)         | 2 (2.9)       | 1 (2.0)        | 0 (0.0)         | 2 (4.5)             | 0 (0.0)          | 7 (4.3)       |
| EST 16                | 8 (5.4)         | 7 (10.0)      | 8 (16.3)       | 5 (10.6)        | 4 (9.1)             | 1 (2.5)          | 33 (20.1)     |
| EST 17                | 3 (2.0)         | 1 (1.4)       | 2 (4.1)        | 0 (0.0)         | 0 (0.0)             | 0 (0.0)          | 6 (3.7)       |
| EST 18                | 3 (2.0)         | 0 (0.0)       | 0 (0.0)        | 0 (0.0)         | 0 (0.0)             | 0 (0.0)          | 3 (1.8)       |
| EST 19                | 3 (2.0)         | 3 (4.3)       | 0 (0.0)        | 0 (0.0)         | 0 (0.0)             | 0 (0.0)          | 6 (3.7)       |
| EST 20                | 3 (2.0)         | 0 (0.0)       | 3 (6.1)        | 0 (0.0)         | 0 (0.0)             | 0 (0.0)          | 6 (3.7)       |
| EST 21                | 2 (1.4)         | 2 (2.9)       | 0 (0.0)        | 1 (2.1)         | 0 (0.0)             | 2 (5.0)          | 7 (4.3)       |
| EST 22                | 33 (22.3)       | 9 (12.9)      | 0 (0.0)        | 10 (21.3)       | 13 (29.3)           | 9 (22.5)         | 74 (45.1)     |
| EST 23                | 7 (4.7)         | 2 (2.9)       | 4 (8.2)        | 1 (2.1)         | 0 (0.0)             | 0 (0.0)          | 15 (9.1)      |
| EST 24                | 10 (6.8)        | 7 (10.0)      | 1 (2.0)        | 5 (10.6)        | 0 (0.0)             | 7 (17.5)         | 30 (18.5)     |
| EST 25                | 11 (7.4)        | 5 (7.1)       | 8 (16.3)       | 3 (6.4)         | 4 (9.1)             | 1 (2.5)          | 32 (19.5)     |
| EST 26                | 3 (2.0)         | 2 (2.9)       | 1 (2.0)        | 1 (2.1)         | 0 (0.0)             | 1 (2.5)          | 8 (4.9)       |

1 n = No. of *E. coli* isolates harboring gene. Values with different subscript letters represent significant differences in the number of isolates in each EST, while values with different superscript letters represent significant differences in the number of isolates among ESTs (p < 0.05).

3.6. Protospacer Match from Spacer Sequences

The protospacers matching plasmids and phages, and sequences are presented in Table 4. Seven and one protospacers were found in CRISPR 1 and CRISPR 2, respectively. Interestingly, protospacer 163, which is associated with virulence due to the tail-associated lysozyme of bacteriophage, had the highest prevalence (20.4%), and it only existed in EST 4 and EST22 (Supplementary Figure S1). Moreover, other protospacers were concerned with the protection of bacteria against the host immune system, such as toll/interleukin-1 receptor domain-containing protein (0.6%), or gene regulation, such as DNA-binding protein (18.5%), DNA-cytosine methyltransferase (7.0%), and *darB*, helicase (0.6%). However, protospacer 177 (7.6%) and protospacer 47 (0.8%) in CRISPR 1 and CRISPR 2 loci, respectively, were domains of unknown function.
Table 4. Protospacers matching plasmids and phages, and spacer sequences in 164 E. coli possessing virulence genes, isolated from bulk tank milk.

| CRISPR Array | Name of Protosparceter Sequences (5′ to 3′) | No. (%) of Isolates | Protosparceter Match |
|--------------|---------------------------------------------|---------------------|----------------------|
| CRISPR 1     | ACATGAATGTCGTTACGACGCGTGTGGTTTTTACC         | 29 (18.5)           | DNA-binding protein   |
|              | TGTACTTACAGCCAAGTCTGCCGACAAAGAGGGAAG |
| 78           | GGAGTGTGGAACGCGCCTGACACTCTCTCC             | 1 (0.6)             | toll/interleukin-1 receptor |
| 81           | TTTGTGCAACCGGCCAAATATGACGCGCTGG            | 11 (7.0)            | DNA-cytosine methyltransferase |
| 107          | AAAACGACTGCGGCTGGTACGCGGCACC               | 1 (0.6)             | darB, helicase |
| 117          | GCTGGTGGCGCTGGCAACGCGACAAAGAGGGAAG | |
| 117          | CGACACCACGGCCGCGCGCGCTGGTCGTTAGCGCGCGCG   | 12 (7.6)            | DUF1380 domain-containing protein |
| 133          | AAAACGACTGCGGCTGGTACGCGGCACC               | 1 (0.6)             | Head decoration protein, Viral protein |
| 162          | TCATAATTACGCCCACTCCGACCCGTACCCGTATGCACCC  | 32 (20.4)           | Tail associated lysozyme |
| 163          | GAAAAATTACGATACGATACGAGACGACAGCTGTTGCCCG   | 1 (0.8)             | DUF1281 domain-containing protein |
| CRISPR 2     | GGAGTGTGGAACGCGCCTGACACTCTCTCC             | 1 (0.6)             | Toll/interleukin-1 receptor |

4. Discussion

Mastitis caused by E. coli is one of the most frequent diseases in dairy cattle resulting from environmental infection, and it is usually characterized by changes in milk composition and quality [1,2]. Although the relationship between virulence factors on bovine mastitis caused by E. coli and its clinical severity has not been fully elucidated, many studies have reported the influence of virulence factors on the establishment of clinically severe infections [6,31]. In this study, 18 out of 33 virulence genes associated with ExPEC were detected, and 89.6% of isolates from normal bulk tank milk carried one or more virulence genes. In particular, fimH, which is associated with the virulence factor adhesin, was detected the most often (80.9%). Guerra et al. (2020) [32] and Zhang et al. (2018) [33] also reported a 100% and 89.9% prevalence of fimH, respectively, indicating its ubiquity among mastitis-causing E. coli isolates. The fimH gene is a bacterial adhesin that helps E. coli bind to host cells and their receptors, and plays a crucial role in causing bovine mastitis by colonizing the mammary glands, resembling the pathogenesis of urinary tract infections [31,34,35]. Other virulence genes of adhesin, such as afa/draBC, sfaS, bmaE, and papG allele 3, were also detected in this study. The prevalence rates of these genes varied from 0.5% to 24.0%, but the presence of these adhesins also implies the facilitated attachment of bacteria onto host cells, helping the colonization of the region and increasing the possibility of mastitis [6,31].

Toxins encoded by virulence genes, such as univicnf, cuf1, cdt, hlyA, and cdtB, are considered essential in the pathogenesis of mastitis following colonization via adhesins. In this study, the most prevalent toxin gene was univicnf (21.9%), while the prevalence of other toxin genes ranged from 1.1% to 2.7%. Lehtolainen et al. (2003) [36] reported that cytotoxic necrotizing factors (CNF), encoded by univicnf, cuf1, and cuf 2, are significantly associated with the persistence of mastitis. Moreover, the potential of CNFs to cause tissue damage or mediate bacteremia can lead to acute mastitis with severe systemic symptoms [37]. Therefore, if whole milk was derived from clinical mastitis rather than from a normal bulk tank, a higher prevalence may be confirmed.

Although the prevalence of the genes iss and traT, which are related to serum survival, was 38.3% and 26.8%, respectively, in this study, several reports have described the absence of a correlation between the presence of these genes and the pathogenicity of mastitis [31,38,39]. Therefore, it is difficult to predict whether the presence of iss and traT genes in E. coli from bulk tank milk may increase the risk of mastitis.

Phylogenetic analysis is increasingly being used as a modern method of determining virulence potential [40]. In this study, the phylogenetic group B1 (64.0%) was the most prevalent, followed by A (20.1%). On the other hand, phylogroups B2 and D, which were reported as highly virulent phylogroups regarding ExPEC [40,41], were detected at 0.0% and 8.5% prevalence, respectively. This result is in accordance with those of previous
studies that phylogroups B1 and A were the most common groups in normal and mastitis milk samples, while phylogroups D and B2 were rarely detected [42]. According to previous studies, mastitis-causing *E. coli* isolates may be related to commensal isolates attaining virulence genes, causing infection in hosts with compromised immune systems [43,44].

*E. coli* contains four CRISPR loci: CRISPR 1, 2, 3 and 4; these are classified as either Type I-E (CRISPR 1 and 2) or Type I-F (CRISPR 3 and 4), depending on the presence of the associated *cas* genes [45]. In this study, 161 (98.2%) of 164 isolates possessing virulence genes were identified to possess CRISPR 1 and/or CRISPR 2, which comprise highly conserved direct repeat sequences with variable spacer sequences [22]. Meanwhile, CRISPR 3 and 4 loci, which possess a lower spacer distribution, were not detected. It was reported that CRISPR 1 and/or 2 loci have been preserved and stationary within *E. coli* over a long period [46], whereas CRISPR 3 and 4 loci are a recent creation [22]. Moreover, the hypervariability of CRISPR loci can be applied in phylogenetic analysis, as in previous reports [21,47]. In particular, Touchon et al. (2011) [22] reported that only the phylogenetic group B2 possessed CRISPR 4, implying that the absence of CRISPR 3 and 4 in this study could be linked to the absence of the phylogenetic group B2. Moreover, the absence of a significant difference in the distributions of CRISPR 1 and 2 in the phylogenetic groups C and D is also suggested to be linked with CRISPR loci and phylogeny.

Because both virulence genes and spacers of CRISPR are acquired by horizontal gene transfer via plasmids and phages [48,49], isolates with different virulence genes can have different distributions in CRISPR content, resulting in different ESTs. In this study, the distributions of EST 4 and EST 22 were significantly higher in isolates harboring *fimH*, *ompT*, *afa/draBC*, and *univcnf*, which play a crucial role in MPEC, compared to isolates carrying the genes *iss* and *traT*, which lack a role in pathogenicity. Therefore, these results suggest that the distribution of spacers may be reflected by the presence of virulence genes, as previously reported [24,50].

The CRISPR system of *E. coli* also functions as a regulatory mechanism and immune system of bacteria [22,51–54]. In this study, three (DNA-cytosine methyltransferase, DNA-binding protein, and helicase) of eight protospacers were associated with gene regulation. Bozic et al. (2019) [55] reported that CRISPR I-E (CRISPR 1 and 2) targets bacterial chromosomes, suggesting its major role in the regulation of endogenous genes. Moreover, protospacer 163, which is linked with bacteriophage tail-associated protein, is homologous to the Type VI secretion apparatus [56], which is associated with the increased virulence of many pathogens [57]. Interestingly, in this study, protospacer 163 was only detected in EST 4 and EST 22, which are ESTs with a significantly higher prevalence in isolates carrying four virulence genes (*fimH*, *ompT*, *afa/draBC*, and *univcnf*).

5. Conclusions

In conclusion, the results of protospacer distribution suggest that CRISPR I-E is linked with gene regulation and pathogenicity in *E. coli*. Moreover, the CRISPR sequence-typing approach helped to clarify and trace virulence potential, by showing significant differences in prevalence based on different virulence genes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12040503/s1, Figure S1: Grouping of 164 isolates into Escherichia sequence types (ESTs) according to spacer contents; Table S1: Names and sequences of all spacers in each CRISPR locus identified in this study.

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References

1. Kaper, J.B.; Nataro, J.P.; Mobley, H.L.T. Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* 2004, 2, 123–140. [CrossRef] [PubMed]

2. Petzl, W.; Zerbe, H.; Günther, J.; Seyfert, H.-M.; Hussen, J.; Schuberth, H.-J. Pathogen-Specific Responses in the Bovine Udder. *Models and Immunopathologic Concepts. Res. Vet. Sci.* 2018, 116, 55–61. [CrossRef] [PubMed]

3. Guerra, S.T.; Dalanezi, F.M.; de Paula, C.L.; Hernandez, R.T.; Pantoja, J.C.F.; Listoni, F.J.P.; Langoni, H.; Ribeiro, M.G. Putative Virulence Factors of Extra-Intestinal *Escherichia coli* Isolated from Bovine Mastitis with Different Clinical Scores. *Lett. Appl. Microbiol.* 2019, 68, 403–408. [CrossRef] [PubMed]

4. Olson, M.A.; Grimsrud, A.; Richards, A.C.; Mulvey, M.A.; Wilson, E.; Erickson, D.L. Bile Salts Regulate Zinc Uptake and Capsule Synthesis in a Mastitis-Associated Extraintestinal Pathogenic *Escherichia coli* Strain. *Infect. Immun.* 2021, 89, e00357-21. [CrossRef]

5. Aslam, N.; Khan, S.-U.-H.; Usman, T.; Ali, T. Phylogenetic Genotyping, Virulence Genes and Antimicrobial Susceptibility of *Escherichia coli* Isolates from Cases of Bovine Mastitis. *J. Dairy Res.* 2021, 88, 78–79. [CrossRef]

6. Fernandez, J.B.C.; Zanardo, L.G.; Galvão, N.N.; Carvalho, I.A.; Nero, I.A.; Moreira, M.A.S. *Escherichia coli* from Clinical Mastitis: Serotypes and Virulence Factors. *J. Vet. Diagn. Invest.* 2011, 23, 1146–1152. [CrossRef]

7. Tenailleon, O.; Skurnik, D.; Picard, B.; Denamur, E. The Population Genetics of Commensal *Escherichia coli*. *Nat. Rev. Microbiol.* 2010, 8, 207–217. [CrossRef]

8. Alfinete, N.W.; Bolukaoto, J.Y.; Heine, L.; Potgieter, N.; Barnard, T.G. Virulence and Phylogenetic Analysis of Enteric Pathogenic *Escherichia coli* Isolated from Children with Diarrhoea in South Africa. *Int. J. Infect. Dis.* 2022, 114, 226–232. [CrossRef]

9. Clermont, O.; Christenson, J.K.; Denamur, E.; Gordon, D.M. The Clermont *Escherichia coli* Phylo-Typing Method Revisited: Improvement of Specificity and Detection of New Phylo-Groups. *Environ. Microbiol. Rep.* 2013, 5, 58–65. [CrossRef]

10. Mora, A.; López, C.; Dabhi, G.; Blanco, M.; Blanco, J.E.; Alonso, M.P.; Herrera, A.; Maman, R.; Bonacorsi, S.; Moulin-Schouleur, M.; et al. Extraintestinal Pathogenic *Escherichia coli* O1:K1:H7/NM from Human and Avian Origin: Detection of Clonal Groups B2 ST95 and D ST59 with Different Host Distribution. *BMC Microbiol.* 2009, 9, 132. [CrossRef]

11. Logue, C.M.; Wannemuehler, Y.; Nicholson, B.A.; Doetkott, C.; Barbieri, N.L.; Nolan, L.K. Comparative Analysis of Phylogenetic Assignment of Human and Avian ExPEC and Fecal Commensal *Escherichia coli* Using the (Previous and Revised) Clermont Phylogenetic Typing Methods and Its Impact on Avian Pathogenic *Escherichia coli* (APEC) Classification. *Front. Microbiol.* 2017, 8, 283. [CrossRef] [PubMed]

12. Leimbach, A.; Poehlein, A.; Vollmers, J.; Görlich, D.; Daniel, R.; Dobrindt, U. No Evidence for a Bovine Mastitis *Escherichia coli* Pathotype. *BMC Genom.* 2017, 18, 359. [CrossRef] [PubMed]

13. Bag, M.A.S.; Khan, M.S.R.; Sami, M.D.H.; Begum, F.; Islam, M.S.; Rahman, M.M.; Rahman, M.T.; Hassan, J. Virulence Determinants and Antimicrobial Resistance of *Escherichia coli* Isolated from Children with Diarrhoea in Bangladesh. *Saudi J. Biol. Sci.* 2021, 28, 6317–6323. [CrossRef]

14. Jung, D.; Park, S.; Raffini, J.; Dussault, F.; Dufour, S.; Ronholm, J. Comparative Genomic Analysis of *Escherichia coli* Isolates from Cases of Bovine Clinical Mastitis Identifies Nine Specific Pathotype Marker Genes. *Microb. Genom.* 2021, 7, 8. [CrossRef]

15. Mojica, F.J.M.; Diez-Villaseñor, C.; García-Martínez, J.; Soria, E. Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. *J. Mol. Evol.* 2005, 60, 174–182. [CrossRef] [PubMed]

16. Barrangou, R.; Fremaux, C.; Deveau, H.; Richards, M.; Boyaval, P.; Moineau, S.; Romero, D.A.; Horvath, P. CRISPR Provides Acquisition of Resistance against Viruses in Prokaryotes. *Science* 2007, 315, 1709–1712. [CrossRef]

17. Hatoum-Aslan, A.; Marraffini, L.A. Impact of CRISPR Immunity on the Emergence and Virulence of Bacterial Pathogens. *Curr. Opin. Microbiol.* 2014, 17, 82–90. [CrossRef]

18. Bonomo, M.E.; Deem, M.W. The Physicist’s Guide to One of Biotechnology’s Hottest New Topics: CRISPR-Cas. *Phys. Biol.* 2018, 15, 041002. [CrossRef]

19. Kim, K.; Yoon, S.; Kim, Y.B.; Lee, Y.J. Virulence Variation of *Salmonella gallinarum* Isolates through SpvB by CRISPR Sequence Subtyping, 2014 to 2018. *Animals* 2020, 10, 2346. [CrossRef] [PubMed]

20. Diez-Villaseñor, C.; Almendros, C.; García-Martínez, J.; Mojica, F.J.M. Diversity of CRISPR Loci in *Escherichia coli*. *Microbiology* 2010, 156, 1351–1361. [CrossRef]

21. Touchon, M.; Rocha, E.P.C. The Small, Slow and Specialized CRISPR and Anti-CRISPR of *Escherichia and Salmonella*. *PLoS ONE* 2010, 5, e11126. [CrossRef]

22. Touchon, M.; Charpentier, S.; Clermont, O.; Rocha, E.P.C.; Denamur, E.; Branger, C. CRISPR Distribution within the *Escherichia coli* Species Is Not Suggestive of Immunity-Associated Diversifying Selection. *J. Bacteriol.* 2011, 193, 2460–2467. [CrossRef] [PubMed]

23. Yin, S.; Jensen, M.A.; Bai, J.; DebRoy, C.; Barrangou, R.; Dudley, E.G. The Evolutionary Divergence of Shiga Toxin-Producing *Escherichia coli* Is Reflected in Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) Spacer Composition. *Appl. Environ. Microbiol.* 2013, 79, 5710–5720. [CrossRef]
Animals 2022, 12, 503

24. Toro, M.; Cao, G.; Ju, W.; Allard, M.; Barrangou, R.; Zhao, S.; Brown, E.; Meng, J. Association of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) Elements with Specific Serotypes and Virulence Potential of Shiga Toxin-Producing Escherichia coli. Appl. Environ. Microbiol. 2014, 80, 1411–1420. [CrossRef] [PubMed]

25. Cady, K.C.; White, A.S.; Hammond, J.H.; Abendroth, M.D.; Karthikeyan, R.S.G.; Lalitha, P.; Zegans, M.E.; O’Toole, G.A. Prevalence, Conservation and Functional Analysis of Yersinia and Escherichia CRISPR Regions in Clinical Pseudomonas aeruginosa Isolates. Microbiology 2011, 157, 430–437. [CrossRef] [PubMed]

26. Ministry of Food and Drug Safety (MFDS). Processing Standards and Ingredient Specifications for Livestock Products; MFDS: Chongju, Korea, 2018.

27. Candrian, U.; Furrer, B.; Höfelein, C.; Meyer, R.; Jermini, M.; Lüthi, J. Detection of Escherichia coli and Identification of Enterotoxigenic Strains by Primer-Directed Enzymatic Amplification of Specific DNA Sequences. Int. J. Food Microbiol. 1991, 12, 339–351. [CrossRef]

28. Chapman, T.A.; Wu, X.-Y.; Barchia, I.; Bettelheim, K.A.; Driesen, S.; Trott, D.; Wilson, M.; Chin, J.J.-C. Comparison of Virulence Gene Profiles of Escherichia coli Strains Isolated from Healthy and Diarrheic Swine. Appl. Environ. Microbiol. 2006, 72, 4782–4795. [CrossRef]

29. Grissa, I.; Vergnaud, G.; Pourcel, C. The CRISPRdb Database and Tools to Display CRISPRs and to Generate Dictionaries of Spacers and Repeats. BMC Bioinform. 2007, 8, 172. [CrossRef]

30. Biswas, A.; Gagnon, J.N.; Bruns, S.J.J.; Finneran, P.C.; Brown, C.M. CRISPRTarget. RNA Biol. 2013, 10, 817–827. [CrossRef]

31. Kaipainen, T.; Pohjanvirta, T.; Shpigel, N.Y.; Shwimmer, A.; Pyörälä, S.; Pelkonen, S. Virulence Factors of Escherichia coli Isolated from Bovine Clinical Mastitis. Vet. Microbiol. 2002, 85, 37–46. [CrossRef]

32. Guerra, S.T.; Orsi, H.; Joaquim, S.F.; Guimarães, J.A.; Loureiro, A.M.; Leite, D.S.; Langoni, H.; Pantoja, J.C.F.; Rall, V.L.M.; et al. Short Communication: Investigation of Extra-Intestinal Pathogenic Escherichia coli Virulence Genes, Bacterial Motility, and Multidrug Resistance Pattern of Strains Isolated from Dairy Cows with Different Severity Scores of Clinical Mastitis. J. Dairy Sci. 2020, 103, 3606–3614. [CrossRef] [PubMed]

33. Zhang, D.; Zhang, Z.; Huang, C.; Gao, X.; Wang, Z.; Liu, Y.; Tian, C.; Hong, W.; Niu, S.; Liu, M. The Phylogenetic Group, Antimicrobial Susceptibility, and Virulence Genes of Escherichia coli from Clinical Bovine Mastitis. J. Dairy Sci. 2018, 101, 572–580. [CrossRef] [PubMed]

34. Connell, I.; Agace, W.; Klemm, P.; Schembri, M.; Marild, S.; Svanborg, C. Type 1 Fimbrial Expression Enhances Escherichia coli Virulence for the Urinary Tract. Proc. Natl. Acad. Sci. USA 1996, 93, 9827–9832. [CrossRef]

35. Langermann, S.; Palaszynski, S.; Barnhart, M.; Auguste, G.; Pinkner, J.S.; Burlein, J.; Barren, P.; Koenig, S.; Leath, S.; Jones, C.H.; et al. Prevention of Mucosal Escherichia coli Infection by FimH-Adhesin-Based Systemic Vaccination. Science 1997, 276, 607–611. [CrossRef] [PubMed]

36. Lehtolainen, T.; Pohjanvirta, T.; Pyörälä, S.; Pelkonen, S. Association between Virulence Factors and Clinical Course of Escherichia coli Mastitis. Acta Vet. Scand. 2003, 44, 203–205. [CrossRef]

37. Wenz, J.R.; Barrington, G.M.; Garry, F.B.; Ellis, R.P.; Magnuson, R.J. Escherichia coli Isolates’ Serotypes, Genotypes, and Virulence Genes and Clinical Coliform Mastitis Severity. J. Dairy Sci. 2006, 89, 3408–3412. [CrossRef]

38. Nemeth, J.; Muckle, C.A.; Lo, R.Y. Serum Resistance and the TraT Gene in Bovine Mastitis-Causing Escherichia coli. Vet. Microbiol. 1991, 28, 343–351. [CrossRef]

39. Blum, S.E.; Leitner, G. Genotyping and Virulence Factors Assessment of Bovine Mastitis Escherichia coli. Vet. Microbiol. 2013, 163, 305–312. [CrossRef] [PubMed]

40. Croxen, M.A.; Finlay, B.B. Molecular Mechanisms of Escherichia coli Pathogenicity. Nat. Reviews. Microbiol. 2010, 8, 26–38. [CrossRef]

41. Croxen, M.A.; Law, R.J.; Scholz, R.; Keeney, K.M.; Wlodarska, M.; Finlay, B.B. Recent Advances in Understanding Enteric Pathogenic Escherichia coli. Clin. Microbiol. Rev. 2013, 26, 822–880. [CrossRef]

42. Liu, Y.; Liu, G.; Liu, W.; Liu, Y.; Ali, T.; Chen, W.; Yin, J.; Han, B. Phylogenetic Group, Virulence Factors and Antimicrobial Resistance of Escherichia coli Associated with Bovine Mastitis. Res. Microbiol. 2014, 165, 273–277. [CrossRef] [PubMed]

43. Müştak, H.K.; Günyaydın, E.; Kaya, I.B.; Salar, M.O.; Babacan, O.; Önay, K.; Ata, Z.; Diker, K.S. Phylo-Typing of Escherichia coli Isolates originating from Bovine Mastitis and Caninepyometra and Urinary Tract Infection by Means Of quadruplex PCR. Vet. Q. 2015, 35, 194–199. [PubMed]

44. Ombarak, R.A.; Hinenoya, A.; Awasthi, S.P.; Iguchi, A.; Shima, A.; Elbagory, A.-R.M.; Yamasaki, S. Prevalence and Pathogenic Potential of Escherichia coli Isolates from Raw Milk and Raw Milk Cheese in Egypt. Int. J. Food Microbiol. 2016, 221, 69–76. [CrossRef] [PubMed]

45. Xue, C.; Sashital, D.G. Mechanisms of Type I-E and I-F CRISPR-Cas Systems in Enterobacteriaceae. EcoSal Plus 2019, 8. [CrossRef] [PubMed]

46. Savitskaya, E.; Lopatina, A.; Medvedeva, S.; Kapustin, M.; Shmakov, S.; Tikhonov, A.; Artamonova, I.I.; Logacheva, M.; Severinov, K. Dynamics of Escherichia coli Type I-E CRISPR Spacers over 42,000 Years. Mol. Ecol. 2017, 26, 2019–2026. [CrossRef]

47. Delannoy, S.; Beutin, L.; Fach, P. Use of Clustered Regularly Interspaced Short Palindromic Repeat Sequence Polymorphisms for Specific Detection of Enterohemorrhagic Escherichia coli Strains of Serotypes O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28, and O157:H7 by Real-Time PCR. J. Clin. Microbiol. 2012, 50, 4035–4040. [CrossRef]
48. Johnson, T.J.; Nolan, L.K. Pathogenomics of the Virulence Plasmids of Escherichia coli. Microbiol. Mol. Biol. Rev. MMBR 2009, 73, 750–774. [CrossRef]
49. Gyles, C.; Boerlin, P. Horizontally Transferred Genetic Elements and Their Role in Pathogenesis of Bacterial Disease. Vet. Pathol. 2014, 51, 328–340. [CrossRef]
50. García-Gutiérrez, E.; Almendros, C.; Mojica, F.J.M.; Guzmán, N.M.; García-Martínez, J. CRISPR Content Correlates with the Pathogenic Potential of Escherichia coli. PLoS ONE 2015, 10, e0131935. [CrossRef]
51. Stern, A.; Keren, L.; Wurtzel, O.; Amitai, G.; Sorek, R. Self-Targeting by CRISPR: Gene Regulation or Autoimmunity? Trends Genet. 2010, 26, 335–340. [CrossRef]
52. Babu, M.; Beloglazova, N.; Flick, R.; Graham, C.; Skarina, T.; Nocek, B.; Gagarinova, A.; Pogoutse, O.; Brown, G.; Binkowski, A.; et al. A Dual Function of the CRISPR-Cas System in Bacterial Antivirus Immunity and DNA Repair. Mol. Microbiol. 2011, 79, 484–502. [CrossRef] [PubMed]
53. Louwen, R.; Staals, R.H.J.; Endtz, H.P.; van Baarlen, P.; van der Oost, J. The Role of CRISPR-Cas Systems in Virulence of Pathogenic Bacteria. Microbiol. Mol. Biol. Rev. MMBR 2014, 78, 74–88. [CrossRef] [PubMed]
54. Aydin, S.; Personne, Y.; Newire, E.; Laverick, R.; Russell, O.; Roberts, A.P.; Enne, V.I. Presence of Type I-F CRISPR/Cas Systems Is Associated with Antimicrobial Susceptibility in Escherichia coli. J. Antimicrob. Chemother. 2017, 72, 2213–2218. [CrossRef] [PubMed]
55. Bozic, B.; Repac, J.; Djordjevic, M. Endogenous Gene Regulation as a Predicted Main Function of Type I-E CRISPR/Cas System in E. coli. Molecules 2019, 24, 784. [CrossRef]
56. Leiman, P.G.; Basler, M.; Ramagopal, U.A.; Bonanno, J.B.; Sauder, J.M.; Pukatzki, S.; Burley, S.K.; Almo, S.C.; Mekalanos, J.J. Type VI Secretion Apparatus and Phage Tail-Associated Protein Complexes Share a Common Evolutionary Origin. Proc. Natl. Acad. Sci. USA 2009, 106, 4154–4159. [CrossRef]
57. Bingle, L.E.; Bailey, C.M.; Pallen, M.J. Type VI Secretion: A Beginner’s Guide. Curr. Opin. Microbiol. 2008, 11, 3–8. [CrossRef]