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The Impact of Plasmid-Mediated Genes on the Virulence of Salmonella Enterica in Human Infections

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Annotation: Salmonella enterica is a significant pathogen responsible for a wide range of human infections, including gastroenteritis, typhoid fever, and septicemia. One of the key factors contributing to its pathogenicity is the acquisition of virulence-associated genes carried on plasmids. These extrachromosomal genetic elements play a crucial role in enhancing the bacterium's ability to invade host cells, evade immune responses, and establish persistent infections. Plasmid-mediated genes encode for various virulence determinants such as toxins, adhesins, and secretion systems, most notably the type III secretion system (T3SS), which facilitates the translocation of effector proteins into host cells. Furthermore, certain plasmids also carry antibiotic resistance genes, which complicate treatment and increase the severity and duration of infections. This review explores the molecular mechanisms by which plasmid-borne genes influence the virulence of S. enterica, with a focus on the IncF and IncI plasmid families known to harbor key virulence and resistance genes. Understanding the dynamics of plasmid

acquisition and expression provides insight into the evolution of highly virulent and multidrug-resistant strains. It also highlights the need for ongoing surveillance and genomic studies to monitor the spread of such plasmids in clinical and environmental settings. Ultimately, targeting plasmid-encoded virulence factors may offer new strategies for combating Salmonella infections, especially in the context of rising antibiotic resistance. This study underscores the importance of plasmid-mediated gene transfer in shaping the pathogenic potential of S. enterica and its implications for public health and clinical management.

Keywords: Salmonella enterica, plasmid-mediated genes, virulence factors, antibiotic resistance, type III secretion system, human infections.

1. Introduction:

Salmonella is a leading cause of bacterial infections and foodborne disease outbreaks in both animals and humans. The genus Salmonella is a member of the Enterobacteriaceae family and consists of two species, S. enterica and S. bongori. Currently, more than 2600 serotypes are recognized in Salmonella [1]. In general, Salmonella serotyping is based on the somatic O and H antigens. Almost 99% of the serotypes associated with disease in warm-blooded animals and humans are members of Salmonella enterica subspecies enteric [2]. The serovars S. Typhimurium, S. Enteritidis, and S. Newport are three leading serovars in human infections in the United States, accounting for nearly 40% of the cases reported. Comparatively, S. Infantis is currently an emerging and the most common serovar in human infections in Europe, alongside S. Kentucky, with multidrug resistance, often conjugated by epidemic plasmids. Phagovar typing is based on the virulence properties of Salmonella and serves as a valuable tool for epidemiological investigations and understanding the evolutionary history and emergence of new strains [3].

Numerous factors can influence virulence, such as pathogenicity islands, plasmid-mediated virulence factors, and single nucleotide polymorphisms. Pathogenicity islands can be integrated into chromosomes or exist as plasmids in Salmonella, as profoundly investigated in S. Typhi, S. Typhimurium, S. Choleraesuis, S. Dublin, and S. Enteritidis. The presence of pathogenicity islands varied largely by serotype, with S. Enteritidis carrying the most and S. Mbandaka, S. Cerro, S. Meleagridis, and S. Havana carrying the least [4].

Salmonella has several plasmid-derived virulence factors that may regulate motility, biofilm formation, and bile salt resistance. S. Typhimurium, S. Choleraesuis, S. I 4,5,12:i:-, and S. Enteritidis each contained the spv operon on hybrid plasmids. Two S. IIIa carried a spv operon with spvD deletion on the chromosome. In total, 12 plasmid types and hybrid plasmids were

identified. IncA/C was frequently associated with S. Newport and S. Agona from bovine, whereas IncFII, IncFIB, and IncQ1 were seen in S. Choleraesuis from swine. Plasmids IncX were found in all S. Kentucky from chicken. Overall, a total of 60 antimicrobial resistance genes, four disinfectant resistance genes, and 33 heavy metal resistance genes were identified [5].

2. Background of Salmonella enterica

Salmonella is a leading bacterial pathogen associated with intestinal infections in both humans and animals. Infected animals are a recognized source of human salmonellosis [6]. S. enterica enterica serotype Typhimurium (hereafter abbreviated as S. Typhimurium) is one of the most widespread serotypes causing infections in a broad range of human and animal hosts. It can cause diseases as diverse as localized enterocolitis in humans and septicemia in cattle and swine. Some of typhoid and paratyphoid fever-causing S. enterica enterica serotypes are exclusively human pathogens and have acquired human-specific virulence traits in various forms including plasmid-borne virulence genes [7,8]. Some Salmonella serotypes can cause more severe diseases in humans through infection by a non-host adapted serotype. Most salmonella infections begin with the bacterium entering through oral ingestion [9]. Salmonella usually infect the epithelial cells of the intestines, where they utilize a variety of systems to survive the nutrient-limiting environment and resist various antibacterial mechanisms such as low pH, bile acids, and antimicrobial peptides. Subsequently, the bacteria can proliferate and spread systemically through lymphatics and the blood stream, leading to typhoid fever, paratyphoid fever or septicemia [10]. Recently, there has been accumulating evidences that the success of Salmonella infection and disease outcome heavily depends on the installation of a diverse arsenal of virulence traits encoded in various forms. Most Salmonella pathogenicity island (SPI) encoded virulence traits were successfully translocated into the eukaryotic host cell by the aid of the Type III secretion system [11]. On the other hand, several virulence factors encoded by plasmids have been conclusively studied, including spvABCD and psl, present in some serotypes like S. Typhimurium, S. Choleraesuis, S. Gallinarum to enhance virulence in systemic infections of various teleost and mammalian hosts [12,13].

2.1. Classification and Characteristics

Salmonella spp. is mainly classified into three groups: S. enterica, S. bongori, and S. salamae. The former group is further grouped into six subspecies, of which S. enterica subsp. enterica is clinically significant and the most important in both human infections and animal diseases, especially in livestock [14]. Because of this perspective, this article will focus on S. enterica. Salmonella enterica is a rod-shaped, non-spore-forming, gram-negative bacterium belonging to Enterobacteriaceae. Like other members of this family, Salmonella uses a wide range of fermentation substrates and has unique biochemical traits. It possesses a type III secretion system, through which it injects proteins into the host cell cytosol to mediate attachment or invasion and survival [15].

It has a genome approximately 5,000,000 bp in length with around 4,700 open reading frames. An important part of its traits is harbored in the large mobile genetic elements, especially pathogenicity islands (PAIs), which are typically present in virulent but absent in avirulent strains [16]. Salmonella pathogenicity islands (SPI-1 to SPI-8) encode virulence factors, including various effectors used to invade and survive in host cells, and genes regulating the activity of the corresponding type III secretion apparatus. Some of S. enterica virulence traits are encoded by plasmids, such as plasmid-mediated pathogenicity islands, self-transferable virulence plasmids, or vectors, which have been targeted for gene delivery technologies [17,18]. Salmonella plasmid virulence plasmids are virulence plasmids belonging to the incompatibility group F. Multiple salmonella serovars possess virulence plasmids, including S. Typhimurium, S. Choleraesuis, S. Dublin, S. Gallinarum, S. C. Java, S. Enteritidis, S. Newport, and S. Typhi. The virulence plasmids are around 50–90kb in size and essentially code for factors mediating the pathogenicity of some salmonella [19]. Comparison of a 50-kb virulence plasmid sequence from S. Choleraesuis demonstrated high homology with plasmids from S. Dublin and S. Typhimurium and 80%

similarity to the S. Typhimurium virulence plasmid. The virulence plasmids are transferable between S. Typhimurium and S. viasventer by conjugation and mediate host-range transfer [20].

2.2. Epidemiology of Salmonella Infections

Salmonella is a leading cause of bacterial infections in animals and humans, necessitating the development of effective intervention strategies. The established 3-strain "non-O157 Shiga toxin-producing Escherichia coli" (non-O157 STEC) performance standards for slaughter and processing plants in 2012 to enhance public health protection against foodborne illness [21]. Additionally, established "Salmonella" and "Campylobacter" performance standards for poultry carcasses in 1996 and 2006, respectively, to enhance public health protection in the National School Lunch Program [22]. In 2012 and 2017, released revised performance standards for Salmonella in certain meat products to reduce this pathogen's presence in products entering commerce. Nevertheless, Salmonella remains the cause of more than 1 million foodborne illnesses, 19,000 hospitalizations, and 380 deaths each year in the U.S [23]. Adequately cooking meat and poultry products and consuming pasteurized eggs will substantially mitigate salmonellosis risk. In 2018, the global burden of foodborne diseases was estimated to be 78 million illnesses, 3.8 million hospitalizations, and 420,000 deaths [24]. Non-typhoidal Salmonella caused 10.9 million illnesses, with an estimated cost of 555 million dollars, 38,612 hospitalizations, and 611 deaths in the USA in 2018. S. Typhi and S. Paratyphi A caused 6 million illnesses, 111,000 hospitalizations, and 117,000 deaths globally, with an estimated cost of $1.36 billion [25,26]. S. Typhi was responsible for 89% of typhoid fever cases and 96% of deaths. It caused 181,000 illnesses and 1,199 deaths in the USA. The estimated cost for S. Typhi was $153.77 million. Recent improvements in evaluation design, methods, and data access and availability are continually enhancing the monitoring of large and small molecular changes in complex food and water environments, populations, and responses within animals, humans, and the earth more comprehensively and structurally [27,28].

3. Plasmids in Bacteria

Plasmids were first discovered from the studies on the F (fertility) factor in Escherichia coli about 50 years ago and have been widely studied in bacteria. Natural plasmids now fall into two broad groups: the R plasmids which carry one or more antibiotic resistance genes and are wide-spread in pathogenic species causing human infection and the virulence plasmids which are transmitted between bacteria and are specific to one or more genus of pathogenic species [29]. Plasmids can be defined as extrachromosomal DNA composed of one or more closed circles of double-stranded DNA (dsDNA) and which can replicate independently of the chromosomal DNA [30,31]. To be called a plasmid, a DNA molecule must satisfy three criteria: Covalently closed circular dsDNA; It must replicate independently of the bacterial chromosome; Some of its genetic information must confer some advantage to the host organism in certain environments. In bacteria, plasmids occur in various shapes and sizes including small or large circles, single or multiple copies [32]. Plasmids carry information for their own reproduction and distribution to daughter cells and for proteins that can be involved in the interaction of plasmids with their host or the extracellular environment. Aside from replication and partitioning genes, plasmids carry several accessory genes such as those involved in antibiotic resistance, pathogenesis, and broad host range [33,34]. The majority of the plasmid dicodes of a broad spectrum of families can be found in gram-negative bacteria such as plasmids of the F, IncQ, IncA/C, IncB, IncI1, Pico, and IncN types, which can replicate in both Escherichia coli and Salmonella enterica. Usually encoding only a single kind of replication initiation protein, all of them, except the IncI1 plasmids, are monotonously distributed in a variety of genera [35]. The transmission and stability of these plasmids can be assured by their own copI/II systems or host-encoded parAB systems. The information concerning the broad spectrum plasmids should shed some light on the discovery of the genetic basis for the wide diversity of virulence and antibiotic resistance of pathogenic strains [36,37].

3.1. Definition and Structure of Plasmids

A plasmid is a small, circular, extrachromosomal DNA molecule that can replicate independently of chromosomal DNA within a cell. Plasmids have essential roles in metabolic processes, growth, and even pathogenesis of bacteria, and their knowledge is necessary for understanding interactions with hosts and epidemiology of disease [38]. This can also be applied to resistance plasmids in Salmonella enterica. Salmonella enterica includes many serotypes which cause the infection of a broad range of hosts. It is a major cause of bacterial zoonoses in humans. The plasmid types prevalent in Salmonella are diverse and highly associated with epidemiology, serotypes and pathogenicity [39].

Plasmids are most commonly classified on the basis of their incompatibility, which is defined as the inability of two plasmids to coexist stably in the same cell type. In general, incompatibility is determined by highly conserved specific regions that are essential for the control of plasmid replication. Plasmid replicons in RapidLAPS are broadly grouped into Inc groups according to their incompatibility. Plasmids of the same Inc group have high nucleic acid similarity and similar replication and control mechanisms. In addition to the broad classification system, there are various typing systems for specific incompatibility groups [40].

The traditional typing methods rely on replicon-specific PCR or hybridization probes. This needs prior knowledge of the sequence of the target replicon. In modern times, with the rapid development of the next generation of sequencing technology, sequencing has been applied to plasmid analysis, which would be the best way if the plasmid sequence is obtained [41,42]. However, sequencing is generally more complicated and cost-demanding. A fast and efficient method would be to characterize the type of plasmids based on the presence or absence of core genes. In order to achieve this goal, 473 plasmids, comprising 31 different replicon types, were detected. The new phylogenetic scheme can be used for rapid typing of novel plasmids [43,44].

3.2. Types of Plasmids

Plasmids are circular strands of DNA extrachromosomal to the bacterial chromosome that can be transferred between bacteria. In addition to housekeeping genes, plasmids often harbor genes involved in drug resistance and virulence [45]. All salmonella plasmids investigated in the past fall into the IncFIB group. Seven plasmids from eight representative Salmonella strains were successfully transferred to the E. coli K-12 strain S17-1, which is deficient in the chromosomal gene int. Plasmid-mediated transfer of multiple antibiotic resistance and virulence genes was investigated by constructing plasmid-free derivatives of FIB plasmid-carrying strains of Salmonella Typhimurium, Typhi, and Enteritidis [46]. Plasmids from these strains conferred transfer of ceftazidime, aztreonam, amoxicillin-clavulanate, and ciprofloxacin resistance. The IncFIB genes mcr-1 and qnrS were harbored by plasmids from S. Typhi. Plasmids from S. Enteritidis revealed phylogenetic relationships with non-Salmonella Enterobacteriaceae. S. enterica Enteritidis strains harbored plasmids containing virulence genes, but no plasmid-mediated virulence carriage was ascertained for S. Typhimurium strains [47]. The genomes of 450 strains of S. enterica isolated from diseased animals were sequenced and subjected to an in-depth comparative genomic analysis. A total of 12 plasmid types were identified, including IncA/C, IncI, IncN, IncHI, IncFII, IncFIB, IncFIA, IncQ, IncX, IncY, IncL/M, and Col. Eighty-five percent of isolates carried at least one plasmid and 65% of isolates carried 2 or more plasmid types [48]. Plasmid type was highly associated with Salmonella serotype and source of the isolates. IncA/C was frequently found in S. Agona isolated from bovine sources, and IncFII and IncFIB were identified in all S. Choleraesuis isolated from swine. IncX was detected in all S. Kentucky isolated from chicken, and 95% of S. Heidelberg isolated from swine carried IncHI [49]. Unexpectedly, IncFIB plasmids from all S. Typhimurium isolates were assigned to pPML12, similar to those from S. enterica Infantis and S. Typhimurium isolated from diseased chickens, instead of pFIB_Ty1 as reported previously. Pathogenic antibiotic resistance genes and virulence genes were identified on plasmids, providing evidence for horizontal transfer of antibiotic

resistance and virulence between Salmonella and other Enterobacteriaceae [50].

3.3. Mechanisms of Plasmid Transfer

Plasmid transfer is a relevant process for the wide distribution of antibiotic resistance genes. The plasmid transfer dynamics of the mobilizable plasmid p3, which carries streptomycin and sulfonamide resistance genes, and its helper plasmid p2 in a complex environment, such as the gastrointestinal tract of mice, were investigated. In laboratory studies, a nonconjugative p3 could be efficiently mobilized by the conjugative helper plasmid p2, and the arginine- and lactose-degrading p3-conjugants did not appear in a medium either lacking lysine or containing 2-deoxyglucose [51]. In studies using a complex environment, mouse-intestinal colonization was more prominent in an S. enterica strain harboring a conjugative p2 and a mobilizable p3 than in a strain bearing only a low copy number native p3 [52,53]. The amount of S. enterica in fecal samples and the amounts of both plasmids in S. enterica not only exponentially increased but also persisted for a longer time after co-colonization with a S. enterica strain harboring both plasmids [54]. The amount of transferable p3, particularly in the presence of a p2 and for S. enterica S. Typhimurium strains, increased at a high dose of pyelonephritis-associated pili [55]. Thus, the transfer of the mobilizable p3 in a complex environment was significantly improved by existing helper plasmids, mating strains, and environmental conditions. Key features also enhance natural competence against KS111 [56,57]. A laboratory study showed a nonconjugative broad-host-range plasmid that relied only on its conjugative helper plasmid within E. coli species, as well as a complex model to understand the effects of small regulatory RNA on plasmid transfer in a complex E. coli community containing both helper and mobilizable plasmids [58,59].

4. Virulence Factors in Salmonella enterica

Salmonella enterica serovar Typhimurium (S. Typhimurium) can cause diseases in a variety of hosts and play an important role in causing enteritis in humans and other mammals [60]. S. enterica have serious threat to chickens as carriers and reservoirs, where it can cause typhimurium and fowl typhoid fever. S. enterica can infect a broad host range with the different pathogenic ability, which is closely associated with a variety of virulence genes [61]. Thus, it is significant to evaluate the virulence factors of S. enterica in infrared spectral analysis. Pathogenicity of Salmonella was often correlated with virulence factors that can promote colonization and survival of Salmonella within hosts. Screen of virulence factors among individual strains is valuable for both the study of pathogenesis and for threat evaluation [62]. However, traditional methods used for filtration of virulence factors are usually time-consuming and complicated. Moreover, due to the cross-contamination between bacteria genes, clear information could not get on the virulence factors of Salmonella stains under family type [63]. Many genes conferring virulence of Salmonella had been reported as potential molecular targets of diseases detection before. The virulence genes could be classified into three categories: virulence-associated invasion affect the intestinal invasion of Salmonella; virulence secretion system contribute the expression, and secretion of many virulence proteins; virulence casailer regulate virulence genes during infection. The technology could allow testing on potentially new Salmonella stains for pathogenicity [64].

To characterize the virulence factors of Salmonella enterica, different strains were initially seeded into different pig organs collected from slaughterhouses. The proxies of intensity and primary chemical arrangement for identification of the family-type and virulence differing strains were obtained by infrared spectral analysis [65]. Targeted intercept of virulence-associated genes was performed with genus-specific primers. PCR and real-time PCR with batch analysis were utilized to obtain accurate information of multi-variation genes. The method could be employed in clinical lab for diagnosis of diseases caused by Salmonella, and in food safety to contamination detection [66]. With the determined virulence spectrum, there may exist specific risk for certain food products concerning the pathogenicity [76]. Moreover, with the general knowledge of virulence genes, it could even be employed prophylactically to obtain safer strains [68].

4.1. Overview of Virulence Factors

About 30% of the open reading frames (ORFs) found in any S. enterica serotype showed no significant similarity to genomics data in databases. The S. enterica subspecies I strains contained more specific ORFs than those found in subspecies II to IV strains despite being less species wide (0.51 versus 0.87%) [69]. This is likely because many strains from subspecies II to IV were represented, and diversity and divergence have likely accumulated with less evolutionary pressure. About half of the ORFs were computably identifiable either as proteins or motifs, while the other half were smaller and poorly identifiable with respect to size and sequence [70]. The first group of proteins included all 87 of the currently assigned S. enterica proteins. Interspecies differences grew greater as the sequence distance from Escherichia coli, a core genome species of S. enterica, increased [71]. The second group was more divergent even though some motifs were otherwise common among species. By definition, the assigned proteins defined the core genome, while those expressed only during animal infection behavior constituted the "virulence core genome" [72]. Using a unique representation of S. enterica strains, new insight was gained into the distribution and identity of virulence factors, giving preference to focusing on plasmid-mediated genes. Here, efforts are made to summarize the current knowledge on the distribution and proposed functions of plasmid-mediated genes, and highlight commonalities and differences within S. enterica that may not be observed with genomic tools used previously [73]. Additionally, other strains subject to additional analyses and which carry traits of serotypes subjects to fewer studies are summarized. S. enterica subspecies I is the most widely studied subspecies in terms of its virulence factors [74]. For the sake of completeness and ensuring other broadly recognized contributors to virulence are discussed, plasmid-mediated virulence factors identified or proposed in strains of other subspecies are also summarized [75].

4.2. Role of Plasmid-Mediated Genes

Plasmid-mediated genes play an important role in the virulence of Salmonella enterica in human infections. The 168 reference S. enterica genomes contained numerous plasmid contigs: 305 contigs were identified as unclassified, but potentially plasmid-encoded, and 166 were assigned to one of six types of known plasmids [76]. All six of these plasmid types were previously shown to occur in strains of the genus Salmonella or other members of the Enterobacteriaceae. These findings suggest that the 171 plasmid-contigs in the 168 genomes may be similar to previously observed plasmid-coding genes [77].

The pSKT1-like plasmid harbored by strain B5068 might contribute to its virulence in humans, but this hypothesis should be verified using genetic manipulation. Genes encoded by the type 084 plasmid have been considered important for pathogenicity in S. enterica serovar Typhimurium, as it was found in pathogenic strains isolated from humans [78]. Importantly, the virulence of both type 084 and 241 plasmids in S. enterica in humans remains unknown. Further investigation is warranted into how conjugative plasmids contribute to trans-phyla virulence plasmid transfer in Salmonella and how these plasmids confer increased virulence to Salmonella in human infections [79].

During the past century, the emergence of antibiotic resistance in all major multidrug resistant (MDR) human pathogens has become a public health threat globally. In recent years, increasing attention has been drawn on the prevalence of plasmid-mediated antibiotic resistance in Salmonella enteric [80,81]. Along with the emergence of virulence plasmids, plasmids harboring antibiotic resistance genes appear to have become popular among some S. enterica strains. In particular, one notable clone of S. Typhimurium CGMR-6 has emerged, which has been implicated in prominent and severe animal and human infections in China and neighboring countries [82]. There is an urgent need to include understanding of the plasmid-mediated antibiotic resistance mechanisms in S. enterica to develop control measures and efficient vaccines in the fight against Salmonellosis [83].

5. Mechanisms of Virulence Enhancement

More than 90% of bacterial infections are caused by biofilms and chronic diseases associated with biofilms are the leading cause of human deaths. Biofilms are composed of aggregates of bacteria embedded in an extracellular polymeric substance matrix. Bacteria in biofilms can have different gene/protein expression patterns as compared with planktonic bacteria. A better understanding of biofilm signaling pathways and their regulators might provide strategies for eradicating biofilms and biofilm-associated chronic infections [84].

Mesophilic Geobacillus species and their unique extracellular processes produce biodegradable bioplastics, in addition to contributing to infectious diseases, spoilage, and corrosion. The genetic bases for biofilm formation, virulence, and degradation of hydrocarbons in Geobacillus genus have not yet been systematically evaluated. The common and diverse features of biofilm formation of five Geobacillus strains are investigated [85]. The mechanisms of biofilm formation of G. kaustophilus are analyzed. Transcriptome-guided assembly reveals that the genome of G. uzenensis consists of one circular chromosome and five plasmids. Comparative genomics highlights the uniqueness of G. uzenensis and G. kaustophilus as well as the diversity of plasmid content across the Geobacillus genus [86].

The evolution of the pathogenicity of Salmonella enterica is a complex process involving the acquisition of several pathogenicity islands. The pathogenicity evolution of Salmonella enterica has also changed the gene structure and content, resulting in evolutionarily divergent serovar [87]. Plasmid-mediated genes have been shown to contribute virulence, resistance, or incapacity of a particular serovar Salmonella enterica. The plasmid sequence prediction presents an overview of the putative genes hosted by the plasmid correction of Salmonella plasmids. Additional analysis sheds light on the roles of the candidate plasmid-mediated genes in the induction of serovar-specific virulence or non-virulence of Salmonella enterica [88,89].

5.1. Adhesion and Invasion

For a successful infection, Salmonella must adhere to and invade the epithelial lining of the intestines, a tightly organized monolayer that separates the lumen from an underlying tissue. This process allows the bacteria to resist the washout by peristalsis and intestinal fluid flow and hide from immuno-competent cells including macrophages [90]. Although a number of Salmonella proteins involved in adhesion and entry have been identified, these proteins need to interact with not only other bacterial components but also host factors to apply their actions successfully [91]. While many microbial factors that S. Typhimurium uses to invade non-phagocytic cells have been identified, relatively little is known about the host factors involved in this process. This genome-wide screen identifies the human genes exploited by S. Typhimurium to facilitate microbial entry into epithelial cells [92]. The set of genes identified in the screen had very modest overlap with an RNAi screen designed to detect host factors associated with invasion mediated by the SPI-1 type III effector SopE [93,94]. The most enriched molecular function category was potassium transport, which is consistent with the current model that the bacteria gain entry by triggering a regulatory cascade linking stimulation of bacterial product entry to potassium secretion and activation of the endocytic pathway. Salmonella injects the SPI-1 type III effector SopB into epithelial cells to manipulate PtdIns-P species to trigger chloride secretion and fluid entry into the GI tract, resulting in diarrhea. However, the involvement of potassium secretion in the entry of Salmonella into epithelial cells was previously unknown [95,96].

Knockdown of KCNJ5 or any other K+ channel regulator significantly decreased internalization of a variety of bacterial strains including wild-type S. Typhimurium, the SPI-1 T3SS mutant, the invasion defective fliC mutant, and EHEC strain EDL933 [97]. Furthermore, the decrease in internalization was rescued by addition of a membrane-permeable potassium salt, suggesting that potassium secretion is a requirement for efficient Salmonella invasion [98]. A plethora of bacterial and host factors have been identified that regulate Salmonella invasion in a cell type-specific manner [99]. However, the genomic screen reveals a number of highly conserved host factors that

mediate Salmonella invasion and highlights potassium secretion by epithelial cells as a requirement for invading Salmonella [100].

5.2. Toxin Production

Salmonella enterica strains were examined. These strains produce diverse toxins, including CDT, Sdo, and RtxA. Different virulence assays proved that these toxins contributed to the virulence of Salmonella enterica in human infections. Salmonella Typhimurium infects and causes significant disease in mice, intermediately pathogenic to humans, and frequently used as a human pathogen model [101]. The virulence plasmid in Salmonella enterica serovar Virchow is associated with a toxin-antitoxin (TA) system, Fisk, and its cognate antitoxin FiskA. The virulence plasmid confers higher pathogenicity and enhanced cytotoxicity toward macrophages. Toxin Fisk precludes translation without an ATG codon, translating a product ~23 kDa longer than the expected size of 166 amino acids [102]. Toxin Fisk is a ribosome-dependent endoribonuclease that cleaves the ribosomal RNA, paralyzing protein translation in vitro. Protein modeling indicates toxin Fisk shares structural similarity with the enterotoxin chinage Z protein (ChzA) that catalyze rRNA modification in vivo. Antitoxin FiskA is a transcript-specific antitoxin only recognizing a small RNA segment containing ribosomal chilling site. Out of the 11 S. enterica serovars screened, only one serovar Typhi strain possesses both toxin and antitoxin genes. To evaluate whether detection of toxin or antitoxin genes can be used as a serpavotype marker, a collection of S. enterica strains that exhibited virulence of wild-type serovar Typhi strains was screened [103].

Various S. enterica strains screened include serovars Agona, Braenderup, Enteritidis, Heidelberg, Infantis, Kentucky, Typhimurium, and serovar Typhi mutants. These strains produced toxin Sdo. All S. enterica strains with plasmids, except biovariants 4 and 2 of serovar Typhimurium, produced toxins CDT and RtxA. High virulent serovars, including serovar Typhimurium and serovar Typhi, frequently exhibited the combination of TAd and TAa genes. Toxins Sdo and RtxA markedly contributed to S. enterica virulence in the human infection model. Other findings showed that TA systems in S. enterica are generally distributed in 35 groups, which are frequently present as 1-4 groups in each strain [104].

5.3. Immune Evasion

Invasive Salmonella can evade the immune surveillance using sophisticated strategies, and could replicate, survive, and cause persistent bacterial infections in hosts without even exhibiting typical clinical symptoms. For example, patients with typhoid fever may carry bacteria in their gallbladder for the rest of their lives. Such infections do not show clinical symptoms but are a potential threat to the host. These asymptomatic carriers presumably act as reservoirs for diverse S. Typhi strains and may act as a breeding ground for new genotypes. S. Typhi chronic infection facilitates gallbladder cancer development in humans [105]. S. Typhimurium involved in persistent infections is also difficult to eliminate, and infected patients often continue shedding these pathogens in the environment, resulting in disease transmission. The innate immune system provides the first line of defense against invading microorganisms by inducing inflammatory and antimicrobial responses [106]. Salmonella has evolved strategies to overcome and adapt to an inflammatory environment. Salmonella may disrupt the tight junctions structure resulting in increased permeability to luminal antigens, degrading the mucosal barrier function. Oral probiotics have been shown to increase intestinal antimicrobial activity and paneth cells, which are responsible for the production of immunoreactive antimicrobial peptide [107]. Salmonella can survive, replicate, and evade the immune attack in macrophages. It has developed and evolved various strategies to achieve cell entry and replication and destroy host immune response via apoptosis, cytokine changes, and metabolic reprogramming. The expression levels of both IL-22 and IL-26 are lower in patients with salmonellosis than in healthy individuals. Phagocytized bacteria may not be totally degraded but survived within the phagosome over the long term in favorable conditions [108]. In response to stress stimuli, only a small fraction may escape to the cytosol and then can be detected by the cytosolic receptor such as Hib. Within the cytosol,

bacterial infection can be eliminated by caspase-1-dependent pro-inflammatory apoptosis, or NF-kB-mediated pro-inflammatory cell death. Bacterial replication is also restricted because Salmonella-induced reactive oxygen species (ROS) which can damage the integrity of protein, lipid, and nucleic acids [109,110].

6. Clinical Implications

Foodborne diseases caused by bacteria are a major public health problem worldwide. Non-typhoidal Salmonella (NTS) is one of the most important foodborne pathogens causing such infections. Several serotypes of NTS are major causes of gastroenteritis, while others are important causes of severe invasive disease. Most salmonellosis is associated with S. enterica serotype Typhimurium or Enteritidis [111]. Invasive non-typhoidal salmonellae (iNTS) are a relatively rare subgroup of NTS that enter the bloodstream causing severe disease, especially in the immunocompromised, and are often associated with high rates of morbidity and mortality [112]. Salmonella enterica is a Gram-negative bacterium that is pathogenic to both animals and humans. S. enterica infections can be asymptomatic or can manifest as mild self-limiting gastroenteritis or as invasive disease involving organs such as the bloodstream, meninges, pregnancy associated tissues, or urinary tract. S. enterica includes more than 2,600 serotypes which differ with respect to virulence, transmission, and the clinical manifestations of infection. S. Typhi and S. Paratyphi are human-restricted and can cause enteric fevers [113].

The emergence and dissemination of multidrug-resistant (MDR) bacteria represent an alarming threat to public health. Particularly troubling are strains resistant to critically important antibiotics such as fluoroquinolones (FQs) and third generation or higher cephalosporins. Salmonella enterica serotypes Typhimurium (ST MT), Heidelberg, and Kentucky are among the most pervasive MDR Salmonella [114]. Despite efforts to monitor and control these pathogens, their global dissemination and continuing emergence of new genotypes raise concern. Indications of viable but nonculturable (VBNC) Salmonella have been observed in marine environments, however, the capacity of ST variants to enter a VBNC state is unknown. Agricultural and aquacultural practices in the Asian and African tropics raise additional concerns and warrant investigation [115].

Bacterial plasmids are small circular pieces of DNA that exist independently of the chromosomal DNA in the cell. The key feature of plasmids is their ability to replicate independently, which is provided by the functions in their replication origin (oriR). Apart from replication functions, many plasmids carry genes that enable their hosts to survive and compete successfully in a variety of environments [116]. These include antibiotic resistance genes, virulence factors, and genes that facilitate the establishment of infection. The virulence phenotypes encoded by these genes range from those that aid the bacteria in persisting outside the host or ensuring successful colonization of the host to those that aid bacteria in resisting military responses from the host or promoting their dispersal [117].

6.1. Diagnosis of Salmonella Infections

The most common laboratory method for the diagnosis of infections with S. enterica is stool culture. Specimens should only be collected from patients who are suspected to have a Salmonella infection during the first 48 h of diarrhea [118]. The methodology consists of several steps that may last from several hours to a couple of days depending on the chosen approaches: (i) stool specimen is enriched in a selective broth, (ii) if turbidity is observed, several loops are streaked onto selective agar plates containing a differentiating agent, (iii) Single colonies with characteristic morphology are purified by subculturing onto nutrient agar, and (iv) the isolated strains are biochemically characterized in order to determine Lactose Simmons' reaction, urease production, hydrogen sulphide production, and the existence of specific serotype antigen polysaccharides [119]. The so far obtained results, which can be confirmed after 2-4 days, do not allow deducing the enteropathogenicity and virulence of the isolated strains. The existence of genes that share a high degree of identity with the virulence plasmid of the well-known virulent strains S. enterica serovars Typhimurium, Enteritidis, and Cholerasuis has previously been shown

for several non-typhoid S. enterica strains isolated from human gastroenteritis [120]. The existence of virulence plasmid genes terD, virE, and invH has also been recently shown for S. enterica strains isolated from tap water in Egypt. Enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis showed that some S. enterica strains isolated from tap water in Egypt are genetically close to strains isolated from human infections [121,122]. The pathogenicity or virulence of a bacterium is typically determined either by its acquired ability to express a specific invasive phenotype due to the possession of plasmid-encoded factors or by chromosomally located virulence genes [123, 124]. Characterization of S. enterica strains isolated from human infections showed that 15 out of 44 strains contained a high molecular mass plasmid with an average size of 46 Kb. The antibiotic resistance pattern of S. enterica strains isolated from human infections against 10 antibiotics. The bands of DNA were transformed into a library using a suitable transformation process to analyze the hybridization [125,126].

6.2. Treatment Strategies

Treatment strategies for infections caused by Salmonella enterica serovar Typhi (S. typhi) have evolved based on the efficiency of antibiotics, antimicrobial resistance (AMR) trends, and the development of vaccines. Based on limited morphological, metabolic, and genetic characteristics, the genus Salmonella is compromised of more than 2600 serotypes. Infections with salmonella result in one of the most prevalent enteric fever illnesses of public health importance that cause high morbidity and mortality [127]. Typhoid fever is an enduring public health problem in developing countries. Infections with S. typhi affect 16 million people across the globe every year, resulting in around 600,000 deaths, with the highest burden of the disease being recorded in sub-Saharan Africa, India, and Southeast Asia. Resistance to multiple antibiotics has been observed in salmonella infections across various countries [128]. Resistance to first-line anti-typhoid drugs has significantly hindered the treatment of enteric fever as fluoroquinolones and azithromycin remain effective against drug-resistant strains. However, treatment with a single fluoroquinolone may prove ineffective when faced with a strain requiring a higher dose for bactericidal activity. Resistance to azithromycin in strains has been noted as well [129]. Therefore, owing to the rising resistance trends, close surveillance and timely intervention strategies, and antibiotics are required to be investigated and refined to treat salmonella-related enteric fever. Prevention and control measures for salmonellosis should focus on all critical points of the food chain. In underdeveloped and developing countries, where healthcare management is compromised, improvement of food hygiene and personal hygiene is very essential to limit the transmission of infections [130]. Young children and immunosuppressed individuals may require vaccination against S. typhi in endemic areas. Currently licensed typhoid vaccines include polysaccharide Vi vaccine, live attenuated oral vaccines, and Typhoid Conjugate Vaccine. In South Asian countries, parenteral ViCPS vaccine and oral Ty2 strain and CVD 1902 vaccine is in use [131,132]. In comparison, in other countries, the Ty21a vaccine is also available. Effectiveness of typhoid vaccines range 47-83% and the duration of immunity is 1-7 years. TCV have also shown high effectiveness. More than 90% of the children vaccinated showed seroconversion at three weeks while no safety concern was reported over two years of follow through. The robustness of such vaccines and increasing incidence of enteric fever demonstrations in the study area demands large scale repetition of the vaccination program [133].

6.3. Public Health Considerations

Foodborne illnesses have become a global public health concern, leading to an economic burden of billions of euros resulting from health-care expenditures and decreased productivity. Salmonellosis cases are mostly attributed to the ingestion of food containing enteric pathogenic bacteria. In humans, Salmonellosis is caused mainly by Salmonella enterica serovars belonging enterica (subspecies I). Salmonella enterica subsp [134]. I is the only subspecies infecting humans and causing systemic disease. Infections with Salmonella enterica subsp. I strains belonging to serovars Typhimurium, Enteritidis, and the monophasic variant of S. Typhimurium occurring in the past decade were globally dominant. In addition, infections with Sydney outbreak variant

strains of S. Enteritidis and monophasic Salmonella paratyphi B have been reported recently [135]. Today and for the last two decades, S. Typhimurium is the leading serovar causing serious infections in humans, including gastroenteritis, bloodstream infections, and systemic infections. S. Typhimurium infections in industrialized countries are often associated with contaminated food products, while infections in non-industrialized countries are often waterborne infections [136]. On the other hand, the use of a regulated bacterium to control the health of growout fish and shellfish is known as biocontrol by aquaculture. The global demand for aquaculture practices has increased recently, emphasizing the need for research on effective biocontrol agents against fish and shellfish diseases. As a large aquaculture country, China's use of antimicrobial agents and antibiotics is prevalent in aquaculture [137,138]. However, the overuse of chemical disinfection and antibiotics may cause aquatic environment pollution and drug-resistant pathogens. Aquaculture biocontrol agents, such as probiotics, competitive exclusion bacteria, phage therapy, and bacteriocin-producing bacterium, provide biological approaches to prevent the introduction and proliferation of aquatic pathogens in industrial aquaculture systems [139]. Many Lactobacillus strains, as probiotics, reduced the pathogenicity of aquaculture pathogens by enhancing the immune system. Aeromonas sp. bacteriophages Ly-Ah and Ly-AT from grass carps showed strong lytic activity and could inhibit the infection of Aeromonas spp. in grass carp fry. For decades, biocontrol agents for aquaculture have been rapidly developed in many countries and are poised for future development [140,141].

7. Research Methodologies

Plasmids are extrachromosomal elements that are capable of autonomous replication and are generally circular double-stranded DNA molecules [142]. The DNA plasmidic information is often encoded by the origin of replication (ori) and a number of replication initiation proteins (Rep proteins). Each plasmid needs at least one ori and one kind of rep to replicate efficiently. Based on their incompatibility groups (Inc), plasmids can be divided into distinct groups [143]. Plasmids of the same compatibility group cannot stably coexist in the same host because they compete for the same replicon and/or Rep proteins. The replication mechanisms of plasmids can be either theta-type or rolling circle [144]. Plasmid replication initiation proteins play a key role in distinct mechanisms of different plasmids. Broad-host-range plasmids, which can replicate in many Gram-negative bacteria, are of great significance in biotechnological applications and have also brought considerable safety implications for biosecurity issues [145].

In this research, the useful culture collection (ULCC), donor strain and entry strains were pre-grown in LB media at 37 °C with shaking overnight. A thousand different Salmonella suspected colonies were stored in 10% glycerol and subsequently screened in terms of pathogenicity by PCR assay [146]. The immunomagnetic beads separation technology was utilized to enrich the S. Typhimurium or S. Enteritidis in chickens' fecal or environment samples, and presumptive Salmonella colonies were selected. The plasmid extraction was performed according to manufacture instruction with some modification (obtain 400 μL of elution buffer and purifying plasmid DNA by 1×TBE gel with 1% agarose gel). 48 identified Salmonella strains were used to determine the virulence locus distribution and the plasmid contents by the PCR assay described here [147]. To test the stability of the virulence plasmid and whether the plasmid transfer can occur in vivo, different strategies were developed. The entry strains were first screened with various enrichment media and selective conditions in order to ascertain where the S. Typhimurium virulence plasmid may reside in vivo, followed by the consumption of either antibiotics in the feed or serum for testing whether conditions of in vivo stress can enhance pSTm transfer. Enterotoxin or H2O2 tolerance experiments were conducted for further investigation of competition in certain environmental conditions [148]

7.1. Experimental Design

The virulence of Salmonella enterica is increased by several plasmid-mediated genes, and the impact of these genes on the virulence of five isolates was comprehensively assessed [149].

Characterization of the plasmid replicon, multi-locus sequence typing, virulence gene presence, and resistance gene screening were conducted using bioinformatic algorithms. The methods of plasmid curing, plasmid absence testing, etc. were used to study the effects of plasmid curing on virulence, biofilm, adhesion, invasion, and Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrum of isolates The results showed that IncFIB plasmids contribute to the virulence of S. enterica through a novel mechanism promoting adhesion. Results revealed that among the five strains, four carried an IncFIB plasmid of 86.017–117.827 kb, and one contained IncF, IncI1, and IncHI1 plasmids [150] One avirulent strain was discovered to have no plasmid based on bioinformatic identification. The IncFIB plasmid was confirmed to promote adhesion of the transconjugant strain. The virulence of the isolate containing the IncFIB plasmids was significantly reduced after curing them, and the absence of plasmids disabled the production of biofilm and secretion of flagella, thus facilitating the understanding of the virulence mechanism of S. enteric [151]. The virulence of S. enterica isolates from different sources was compared, and the virulence factors and antimicrobial resistance factors of a highly virulent strain were further explored using whole genome sequencing technology [152]. Compared with the low virulence strain, the phylogenetic analysis results indicated that the pathogenicity of the highly virulent strain originated from the acquisition of a large number of virulence factors as well as the deletion of non-pathogenicity determinants. Simultaneously, it was found that a high virulent strain harbored a broader antimicrobial resistance gene profiles than low virulent strains. This study elucidated the virulence differences of SMT strains and acquired insights into the in-depth virulence mechanism of Salmonella pathogenicity [153].

7.2. Molecular Techniques

Molecular techniques help to understand the genetic basis of pathogenicity. Pathogenicity-related genes can be divided into two classes: (1) essential pathogenicity factors, including those coded for by plasmids or chromosomes, and (2) accessory pathogenicity genes, usually found on the chromosome. Many virulence factors often produce a competitive advantage against their nonvirulent counterparts, which can be specifically traced back to low probabilities of homologous recombination events with non-pathogenic strains [154].

A collection of virulence traits of Salmonella confirms that many virulence genes of Salmonella strains in the human population have undergone changes with high probability [155]. This is consistent with the absence of such traits in non-pathogenic strains or serovars that are poorly associated with human disease. Many data suggest that genes required for basic functions do not show a high variability in position due to high levels of synapobiosis that prevent serious alterations. This allows to check conservation rates of pathogenicity-related genes in phylogenetically closely related strains. To assess the presence and variability of plasmid-encoded virulence factors in bacteria, a high throughput sequencing procedure was applied to a single locus analysis of the virulence gene SPvB. A single virulence region on pSLT-like plasmids of all strains under investigation was revealed [156].

7.3. Data Analysis

Many Salmonella enterica strains isolated from human infections were classified as serovars Typhimurium, Enteritidis, and Paratyphi B. In a previous molecular epidemiological study, these lineage-specific plasmids had not been detected, and only the two plasmids, pO108 and pF1-IR2, were identified as common between Salmonella enterica strains isolated from human infections [157].

Two high-quality pSEN001 and pCer27 plasmids were assembled from the seven plasmidotus strains and used as the queries for an integrative and comparative genomic analysis of all 450 S. enterica plasmids. The results showed that these two novel plasmids contained several distinct replicons, such as pCol157-5, pYwL, pQ1812, pS0463, p0AB404, pADI, pZK3_B1_O7, and other broad-host-range plasmids [156]. Phylogenetic trees showed that many similar plasmids shared a common evolutionary pathway and were stably maintained in different S. enterica

serovars. The findings indicate the important role of plasmid-mediated genes on the virulence of S. enterica strains in human infections [156,157]. The similarity of pSEN001 and pCer27 to the other plasmids suggested that several virulence factors associated with human infections emerged before or during the divergence of serovars Typhimurium, Enteritidis, and Paratyphi B. In contrast, these factors acquired either recently or in an intra-serotype fashion in CipR and CefR plasmids [150,157].

In summary, many virulence plasmids acquired during the divergence of the three lineages were confirmed in Salmonella enterica in humans. The results also provide an important framework to better understand the evolution and pathogenic mechanisms of Salmonella enterica in human infections. Future experiments will examine other plasmids, including those within the pYwL, pCer27, and p0AB404 families [153,157].

8. Conclusion

This article summarizes current understanding of the distinct mechanisms of virulence plasmids initially identified in pathovars of Salmonella enterica. Recent work has shown that some of these plasmid-mediated genes impact the virulence of nontyphoidal serovars, while further characterization of genes involved in regulating their virulence expression and genes that impact the expression of host cell invasiveness are presented. One feature of a virulence gene that is often overlooked is the possibility that a genetic element other than a chromosomal gene might be allelelic and impact virulence or pathogenesis as a copy number change. This idea has ramifications since it implies that the presence of a nonfunctional nucleotide changes is an additional risk of pathogenesis. Current work investigates whether the Rck virulence protein of Salmonella enterica serovar Choleraesuis can be present in E. coli, an organism that does not have the chromosomally encoded Rck gene. The presence of rck in a plasmid vector is being examined to see whether it can impart protection from complement. Further comparisons of pathogenic E. coli strains with pathogenic S. enterica have revealed the presence of rck both chromosomally and on plasmids. These plasmid sequences were type II plasmid sequences common in E. coli and highly unlike those found in Salmonella pathogenicity islands. These results are being extended with serovars that do not contain rck. Recent results of a Crp-mediated regulatory cascade are reshaping understanding of the serovar Typhimurium regulation of SPI1 genes. Initial studies focused on the regulatory networks impacted by Crp after examining strains with mutations in crp and pfkA. This approach has resulted in several candidate genes for Crp-dependent regulation of Salmonella enterica virulence genes. Further genetic analysis of these candidates and their regulatory interactions with crp, cAMP, and PfkA is underway. With parallels to the global control of motility and virulence gene expression in pathogenic strains of E. coli now emerging, a more straightforward picture of this significant regulatory interaction is emerging.

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