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Roles of various virulence and resistance genes associated with *Salmonella* and methods of their identification

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Abstract

Salmonellosis is an infectious disease affecting human and animals. The virulence of *Salmonella* is a function of chromosomal and plasmid factors, many genes code for these factors. *Salmonella* genes can be classified as core (housekeeping) genes and accessory genes. The core genes of a species are those genes found in (nearly) all known members of the species and they include mostly genes that are necessary for the cell to survive and grow, these include gene encoding enzymes which function in biosynthetic pathways. Genes in the accessory genome are those unique to particular strains and are mainly in the following groups: genomic islands including *Salmonella* Pathogenicity islands (SPIs), prophages, insertion sequences.

Keywords: Genes, Pathogenicity, Resistance, Salmonella, Virulence.

Introduction

Salmonella is a gram negative, non-lactose fermenting and non-spore forming, facultatively anaerobic, rod-shaped bacterium that is actively motile with the exception *Salmonella enterica* sub. *enterica* ser. Pullorum and *Salmonella enterica* sub. *enterica* ser. Gallinarum. They are also non-capsulated with the exception of *Salmonella* Typhi/Paratyphi (Andrew, 2004) and belong to the family *Enterobacteriaceae*. *Salmonella* is classified into two species, *Salmonella enterica* and *Salmonella bongori* (Reeves *et al.,* 1989). In turn, *S. enterica* is divided into six subspecies I, II, IIIa, IIIb, IV, and VI known as *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica* and *houtenae* respectively. The subspecies I has, to date, over 2,500 serovars, (Porwollik *et al*., 2004; Dana *et al.*, 2015) which are commonly associated with infections of birds and mammals, including humans. The other subspecies are primarily isolated from infected cold-blooded vertebrates. Salmonellosis results in varieties of clinical syndromes, the most common include gastroenteritis, with the organism proliferating in the submucosae of the intestine, and diarrhea sequel to inflammation and probably toxins (Rafael & Josep, 1999). On the basis of phylogenetic analyses, different factors have been reported to influence the existence and persistence of *Salmonella* species in animals, such as cross-contamination among animals, environment and feed (Karen *et al*., 2013).

Salmonella species have virulence and pathogenicity which depend on combination of chromosomal and plasmid factors (Oliveira *et al*., 2003; Ahmed *et al*., 2017), and many studies have identified genes that code for these factors. The outer structures of the bacteria, such as fimbriae constitute some of the virulence factors (Porwollik *et al*., 2004; Dana *et al.*, 2015). The long polar fimbriae (*lpf operon*), aggregative fimbriae (*agf operon*), and *Salmonella*-encoded fimbriae (*sef* operon) respectively aid the bacteria adherence to Peyer's patches and M cells, aid in initial colonization of the host intestine and improve survival rate of the organism via induction of bacterial self-aggregation and enhance a better interaction between the bacteria and the macrophages (Baumler *et al*., 1996; Collinson *et al*., 1996).

This review is aimed at evaluating the roles of virulence and resistance genes associated with *Salmonella* and the various methods of identifying these genes.

Classification of *Salmonella* genes

Salmonella genes can be classified as Core (housekeeping) and Accessory genes. Housekeeping (Core) genome is the gene compliment common to specific group of bacteria such as *Salmonella*. This has been reported to include genes that are vital for the cell to survive and replicate in the host cell as shown in Table 1, these include genes that encode enzymes which function in biosynthetic pathways (Table 2). Almost all genes identified on the linkage map of *S*. *Typhimurium* LT2 are part of the core genome, majority of which are found in both *Salmonella* and *Escherichia coli*. As initially confirmed by genetic and physical maps and lately by sequencing (McClelland *et al*., 2001; Liu *et al*., 2009), usually, the core genomes are extremely conserved both in sequence and in synteny (gene order). Core genes of a species are those genes found in all known members of the species such as *Salmonella* (Tettelin *et al*., 2005). Genes found to be present across bacterial genomes of the same species (or genus) are almost always conserved. A fraction of these genes– those conserved in all (or most) of the genomes of a given bacterial taxonomic group is called the 'core-genome' of that group. The core-genome can be identified either within a genus or species (Malorny, 2011) and can be used to identify the variable genes in a given genome (Adékambi *et al*., 2011). Furthermore, the conserved genes generally develop more slowly, and can be used to determine similarities among bacterial isolates (Urwin & Maiden, 2003). The ribosomal genes are involved in protein synthesis which is essential for the survival of all cells, and hence, their structure cannot change much because of their functions (Sacchi *et al*., 2002). Consequently, 16S rRNA genes are highly conserved among isolates belonging to the same bacterial species (Lukjancenko *et al*., 2010). For the purposes of species identification, genes such as *sod*A or *rpo*B have been suggested as replacement for 16S rRNA (Clerck & Vos, 2004), although, a single gene cannot always reflect the subtle differences between genomes of the same species. It has been reported that the study of phylogeny is based on seven housekeeping genes which are peculiar to each bacterial species. For *Salmonella*, these are: *aro*C, *dna*N, *hem*D, *his*D, *pur*E, *suc*A and *thr*A (Anon, 2018).

The core genes have two categories; the first being a small group of highly variable genes, and the second is a large group of genes which are highly conserved. The former has higher amino acid sequence variations than nucleotide sequence variation, whereas the opposite is the case for the more conserved core genes. This indicates that, for the highly conserved portion of core genes, there is a selection against mutations leading to changes in amino acid sequence, while on the contrary, there is positive selection for amino acid changes in highly variable core genes. Therefore, the variation of amino acid sequence in highly variable core genes might be due to an increase in positive selection at some sites. Although there is need to confirm the importance of this phenomenon by further analyses, this could be a selective pressure to alter the surface proteins to avoid host immune response.

The several other genes apart from the ones described above are often unique to a single *Salmonella* lineage or a small evolutionary group of *Salmonella* lineage and are referred to as accessory genome. The term core or accessory genome is not absolute; genes may be described as being core or accessory genome depending on the strains that are compared. When two *Salmonella* strains are compared, the core genome includes genes that can be found in both organisms. They may differ from one another in host range, kind and severity of disease they cause, and in metabolism. The phenotypic divergences between the distinct *Salmonella* lineages are presumed to be due primarily to differences in accessory genome (Liu *et*

Table 1: *Salmonella* virulence genes

Genes	Mnemonic	Description/function	References
*Org*A	Oxygen-regulated gene	Host recognition/invasion	Baumler *et al.,* 1998
*prg*H	*pho*P-repressed gene	Host recognition/invasion	Baumler *et al.,* 1996
Span	Surface presentation of antigen	Entry into non-phagocytic cells, killing of macrophages	Behlau & miller 1993
*tol*C		Host recognition/invasion	Galan & Curtiss 1989
*sit*C	Starvation inducible	Iron acquisition	Chen *et al.,* 1996
*msg*A		Survival within macrophage	Gulig *et al.,* 1993
*spi*A		Survival within macrophage	Janakiraman & Slauch, 2000
*sop*B		Host recognition/invasion	Jones & Falkow, 1994
*lpf*C		Host recognition/invasion	Haghjoo & Galan, 2004
*sif*A		Filamentous structure formation	Behlau & miller, 1993
*spv*B		Growth within host	Gunn *et al.,* 1995
*ata*A	Attachment	attP22 I; attachment site for prophage P22	Chen *et al.,* 1996
*atb*A	Attachment	attP27 I; attachment site for prophage P27	Chen *et al.,* 1996
*atb*B	Attachment	attP27 II; second attachment site for prophage P27	Ochman *et al.,* 1996
*atr*G	Acid tolerance response	Defective in both pre- and post-acid shock-induced acid tolerance	Ochman *et al.,* 1996
*atr*R	Acid tolerance response	atbR; constitutive acid tolerance	Parsot, 1994
Attn	Attachment	Attachment site for prophage in *S. Montevideo*	Miller *et al.,* 1989
*flg*A,B,C,D,E,F,G	Flagella	flaFI; Flagellar synthesis; P-ring formation of the flagellar basal body protein, hook-associated protein	Skyberg *et al.,* 2003
*mut*G,H,L,S,U,Y	Mutator	Increased frequency of mutation in host chromosome, Mutations inactivate methyl-directed mismatch repair.	Skyberg *et al.,* 2003
*mvi*N,S	Mouse virulence	Affects the virulence of cells in mice	Stone & Miller 1995
*inv*A,D,E,F,G,H	Invasion	Invasion-related function, affects invasion but not attachment to cultured epithelial cells, sequence similarity to proteins for protein translocation	Haghjoo & Galan, 2004

al., 2009). Mainly, the genes in the accessory genome are found in the following groups: genomic islands including *Salmonella* pathogenicity islands (SPIs), insertion sequences prophages. Up to date, 17 different SPIs have been identified to encode the most salient virulence phenotypes, that is, host-cell invasion and intracellular pathogenesis (Helena *et al*., 2012). SPI-7 is the largest of these islands and it is reported to be found within the genomes of *Salmonella* Paratyphi C, *Salmonella* Typhi and some strains of *Salmonella* Dublin. It has a length of 120 kb and encodes important virulence functions, including the type 4B pili and major virulence antigen (Vi) (Helena *et al*., 2012). *Salmonella* pathogenicity island 1 (SPI1) is important in host invasion (bacterial adhesion and penetration of the epithelial cells of the intestine), while SPI2, SPI3, and SPI4 play significant roles in growth and survival of bacteria

Table 2: *Salmonella* genes encoding enzymes which function in biosynthetic pathways

Genes	Mnemonic	Description/function	Reference
*acc*A		Acetyl-CoA carboxylase	Baumler *et al.,* 1998
*ace*A	Acetate	Growth on acetate or fatty acids; isocitrate lyase	Baumler *et al.,* 1996
*ace*B	Acetate	Growth on acetate or fatty acids; malate synthase	Behlau & miller, 1993
*ace*F	Acetate	Acetate requirement; pyruvate dehydrogenase (pyruvate lipoate oxidoreductase)	Chen *et al.,* 1996
*aci*A	Acid inducible	pH regulated gene; acid inducible	Galan & Curtiss 1989
Ack	Acetate kinase	Acetate kinase (ATP: acetate phosphotransferase	Gulig *et al.,* 1993
*ahp*C	Alkyl hydroperoxide	Alkyl hydroperoxide reductase C22 subunit	Janakiraman & Slouch, 2000
*ahp*F	Alkyl hydroperoxide	Alkyl hydroperoxidereductase, F52a subunit	Jones & Falkow, 1994
Alas	Alanine	Alanine tRNAsynthetase	Jones & Falkow, 1994
*apb*A		Alternative pyrimidine biosynthetic pathway; synthesis of thiamine in presence of exogenous purines	Skyberg *et al.,* 2003
*ara*A,C	Arabinose	L-Arabinose isomerase, Regulatory gene for arabinose catabolic enzymes	Stone & Miller 1995
*arg*A,B	Arginine	amino acid acetyltransferase, N-acetyl- [gamma]-glutamate kinase	Stone & Miller 1995
*aro*E,T	Aromatic	5-dehydroshikimate reductase Transport of tryptophan, phenylalanine, and tyrosine	Skyberg *et al.,* 2003
Asn	Asparagine	Asparagine synthesis	Skyberg *et al.,* 2003
*che*B	Chemotaxis	cheX; chemotaxis; bifunctional monomeric protein; C-terminal gamma-carboxyl methyl esterase and N-terminal transferase	Galan & Curtiss, 1989
Caps	Capsule	Capsular polysaccharide synthesis	Chen *et al.,* 1996
*cbi*A		cobI; synthesis of vitamin B12 adenosyl cobamamides precursor	Skyberg *et al.,* 2003
*cys*L	Cysteine	Resistance to selenite	Stone & Miller, 1995
Dcm		DNA cytosine methylation	Skyberg *et al.,* 2003
*dna*E,Q,X,Y	DNA	DNA synthesis	Skyberg *et al.,* 2003
*hem*A,B,C,D,E,G,H,K,L,M	Heme	Glutamyl t-RNA dehydrogenase, Heme deficient, urogen I synthase, Accumulation of uroporphyrin III, Protoporphyrinogen oxidase; putative	Parsot, 1994
Hin	H inversion	vh2; flagellar synthesis; regulation of flagellin gene expression by site-specific inversion of DNA	Parsot, 1994
*his*A,B,C,D,F,G,H,I,M,P,Q	Histidine	N-(5'-phospho-L-ribosylformimino)-5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide isomerase	Skyberg *et al.,* 2003
*mot*A,B	Motility	Non-motile but flagellate	Skyberg *et al.,* 2003

within the host which result in the systemic phase of disease (Sandra *et al*., 2000). Insertion sequences (IS) are genetic elements that can plug replicates of themselves into distinct sites in a genome. They can also mediate rearrangements of chromosome such as DNA deletions, circular DNA fusion and/or inversions. Alteration in the expression of adjacent genes can also occur via insertion sequences (Kleckner, 1983). Insertion sequence IS200 is a transposable element of some 700 bp which has been shown to be confined to salmonellae with the exception of *S*. Agona, while *E. coli* insertion sequences IS1-4 are not present in *Salmonella* (Isidre *et al*., 1990).

Plasmids encoded virulence has been postulated to aid pathogenicity in enteric bacteria such as *Escherichia coli*, *Yersinia* spp. and *Shigella* spp. Historically, the existence of plasmid-borne virulence genes in *Salmonella,* was dated to 1982, but currently it has been proven that virulence plasmids contribute little to pathogenesis in *Salmonella* than in the aforementioned bacteria. It has been reported that only a few serovars of *Salmonella* belonging to subspecies *enterica*, particularly those showing host adaptation, harbour virulence plasmids. The sizes of these plasmids range from 50 to 90 kb and have been called "serovar-specific plasmids" (Guiney *et al*., 1994). Not every isolate of a plasmid-bearing serovar carries the virulence plasmid (Boyd & Hartl, 1998). Multidrug resistant (MDR) *Salmonella* has been increasing in the recent time and become a major health problem especially in developing countries (Butaye *et al*., 2006). Drug resistance genes had been reported to play vital roles in conferring drug resistance to bacteria including *Salmonella* (Rungtip & Pawin, 2009). There are many mechanisms through which drug resistance mediate in different *Salmonella* serovars. for instance, quinolones resistant *Salmonella* has resistance that is due to single point mutation in the quinolone-resistance determining region (QRDR) of the *gyr*A gene which occur in the nucleotides 67 to 122 (Guerra *et al*., 2003). These MDR *Salmonella* pose direct health hazard to human and animals when the multidrug resistance phenotype harbored in their genes interferes with the efficacy of antimicrobial treatment. It may also occur indirectly when resistance is transferred to other human or animal pathogens.

The functions of *Salmonella* virulence and resistance genes can be summarized as shown in the tables below (Tables 1, 2 and 3)

Methods of identification of virulence and resistance genes

Current protocols for the identification of bacterial genes may utilize a variety of different fingerprinting- or sequence-based methods, either alone or, more often, in combination. These techniques are constantly evolving to embrace new methodologies that provide both greater accuracy for identification and higher sample throughput. Examples of some of the most widely used techniques are provided below:

Fingerprinting-based methodologies

Currently, the most commonly used methods for bacterial gene identification are fingerprinting techniques. Some of these techniques such as amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA, and repetitive element PCR (rep-PCR) make use of PCR to amplify several copies of short DNA portions, employing specific sets of primers (Versalovic *et al*., 1994; Cocconcelli *et al*., 1995; Vos *et al.*, 1995; Lin *et al*., 1996; David *et al*., 2008). The techniques are built to take advantage of DNA polymorphisms in related organisms that may accrue as a result of a variety of evolutionary mechanisms. Unique sets of primers for more than one organism are utilized in multiplex PCR to identify more than one microbe simultaneously in a mixed sample. These sets of primer can be separated on the basis of amplicon size (Settanni & Corsetti, 2007; David *et al*., 2008). Riboprinting is another form of fingerprinting technique which utilizes a specific probing technique to identify differences in gene patterns (mainly rRNA genes) between strains and species (David *et al*., 2008). Denaturing-gradient gel electrophoresis uses PCR products which are obtained from bacteria deoxyribonucleic acid (DNA) making use of primers for 16S ribosomal RNA gene (or any other specific molecular marker). The products are then electrophoresed on a polyacrylamide gel that contain a DNA denaturant like mixture of urea and formamide (Muyzer *et al*. 1993). Temperature-gradient gel electrophoresis uses the same principle as Denaturing-gradient gel electrophoresis Other than a temperature gradient that is employed as denaturant (Gurdeep & Rajesh, 2011).

Single-strand conformation polymorphism is another form of fingerprinting technique. Here, the PCR products are denatured using electrophoresis, single-stranded DNA fragments are then separated on a nondenaturing polyacrylamide gel (Muyzer *et al*. 1993). Amplified ribosomal DNA restriction analysis employs variations that occur in DNA

Table 3: Drug resistance *Salmonella* genes

Genes	Mnemonics	Description/function	Reference
*amp*C	Ampicillin	beta-Lactamase; penicillin resistance	Cesco *et al.,* 2008
*amp*D	Ampicillin	beta-lactamase regulation; putative signaling protein	Cesco *et al.,* 2008
*cor*A,B	Cobalt resistance	Magnesium transport; cobalt resistance (high level)	Prager *et al*., 2003
*sap*A,B,C,D,E,F,I,J,K		Resistant to antimicrobial peptides melittin and protamine	Janakiraman & Slouch, 2000
*azi*A	Azide	Resistant to sodium, L-methionine	Guo *et al*. 2000
*chl*F	Chlorate	Resistance may be part of *moe* operon	Murugkar *et al*., 2003
*chl*G	Chlorate	Resistant to Chlorate; affects nitrate reductase, tetrathionate reductase, chlorate reductase, and hydrogen lyase	Chen *et al.,* 1996
*cys*A, B	Cysteine	Sulfate-thiosulfate transport; chromate resistance	Gulig *et al.,* 1993
*spc*B,C	Spectinomycin	Non-ribosomal resistance, Low-level resistance plus auxotrophy	Chen *et al.,* 1996
Tlr		Thiolutin resistance; P22 development at high temperature	Gulig *et al.,* 1993
*ton*B	T-one	chr; regulates levels of some outer membrane proteins; resistance to ES18, affects iron transport	Cesco *et al.,* 2008
*tpp*B	Tripeptide permease	Resistant to alafosfalin; tripeptide permease	Murugkar *et al*., 2003
*trp*R	Tryptophan	Resistance to 5-methyltryptophan; depression of tryptophan enzymes	Skyberg *et al.,* 2003
*qnr*S	Quinolone	Resistant to Fluoroquinolones	Raufu *et al.,*2013

sequence present in PCR-amplified 16S ribosomal ribonucleic acid (rRNA) genes (Gurdeep & Rajesh, 2011).

Sequence-based methodologies

Multilocus sequencing is one of the newest and, till date, one of the most powerful methods developed to identify bacteria genes. The methodology of this technique is similar to 16S comparisons of ribosomal RNA gene sequence. However, the fragments of many core genes are each sequenced, combined and linked together into one long sequence that can be compared with other sequences. Housekeeping genes are generally defined as encoding for proteins that carry out essential cellular processes. A few examples include *gyr*B (B subunit of gyrase gene), *rpo*A and *rpo*B (α and β subunits of RNA polymerase gene), and the gene that code for an enzyme important in DNA repair, that is, *rec*A (Zeigler, 2003). Housekeeping-gene loci are present in most cells and tend to be conserved among different organisms. As a result, general-purpose primers can be designed that will work using PCR to amplify the same genes.

The two multilocus sequencing techniques that are currently in use include: multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA). MLST is a well-defined approach that uses a suite of 6 to 10 genetic loci, with appropriate primers for each locus to allow PCR amplification and sequencing of the products (usually 400 to 600 base pairs) (Maiden *et al*., 1998). The resulting concatenated sequences can then be compared with a curated database of sequences for the same gene. The result provides a high-resolution identification of an individual gene that may reveal close evolutionary relationships among individual genes. This technique has proved useful in epidemiological studies, making it possible to track the outbreak of virulent genes (Cooper & Feil, 2004). So far, MLST, and the robust databases that have been created for it, has been applied only to a relatively small number of common genes, using highly prescribed conditions for each organism, both for PCR primers and for database analysis.

MLSA also involves sequencing of multiple fragments of conserved protein encoding genes, but it uses a more ad hoc approach to choosing the genes for comparative analysis. A smaller subset (≤6) of genes or loci is typically used in MLSA than is used in MLST (Gevers *et al*., 2005). MLSA is typically used to identify genes in the broader context than MLST. As typically applied, it does not have the analytical capacity to detect the very minor changes in sequence patterns that are useful in epidemiologic studies. At present, MLSA is limited by a lack of standardization, and no central databases are available.

Conclusion

Salmonella has a number of different genes which confer virulence or drug resistance to this organism. These genes can be classified as core genes which are housekeeping genes common to all serovars or accessory genes which are specific to each serovar of *Salmonella*. Plasmid encoded virulence has been postulated to aid pathogenicity in enteric bacteria, as seen in *Escherichia coli*, *Yersinia* spp. and *Shigella* species, however, plasmid coded virulence is not as important in *Salmonella* as the other bacteria mentioned above.

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