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Original Article

Identification and bioinformatic analysis of invA gene of Salmonella in free range chicken

Identificação e análise bioinformática do gene invA de Salmonella em frango caipira

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Abstract

Salmonella is a serious cause of the health issues in human and animal worldwide. Salmonella has been isolated from different biological samples and it considers as the key role in induction of inflammation of gastrointestinal tract which in turn cause diarrhoea in different species. To further understand the involvement of Salmonella in contaminating and infecting fresh eggs and meat of free-range chicken. This study aimed to establish the microbiological and molecular detections of Salmonella in the cloaca of the free-range chicken and to identify predicted biological functions using Kyoto Encyclopedia of Gene and Genomic (KEGG) pathways and protein-protein interaction. Cloacal swabs were collected from free range chicken raised in the local farm in Duhok city. The isolates were cultured and biochemical test performed using XLD and TSI, respectively. Molecular detection and functional annotation of invA gene was carried out using Conventional PCR and bioinformatics approaches. The present study found that Salmonella was detected in 36 out of 86 samples using microbiological methods. To confirm these findings, invA gene was utilised and 9 out of 36 Salmonella isolates have shown a positive signal of invA by agarose gel. In addition, bioinformatic analysis revealed that invA gene was mainly associated with bacterial secretion processes as well as their KEGG terms and Protein-Protein Interaction were involved in bacterial invasion and secretion pathways. These findings suggested that invA gene plays important role in regulating colonization and invasion processes of Salmonella within the gut host in the free range chicken.

Keywords: invA, Salmonella, free range chicken, conventional PCR.

Resumo

A salmonela é uma causa séria de problemas de saúde em humanos e animais em todo o mundo. Salmonella tem sido isolada de diferentes amostras biológicas e é considerada como o principal papel na indução da inflamação do trato gastrointestinal que por sua vez causa diarreia em diferentes espécies. Compreender melhor o envolvimento da Salmonella na contaminação e infecção de ovos frescos e carne de frango caipira. Este estudo teve como objetivo estabelecer as detecções microbiológicas e moleculares de Salmonella na cloaca da galinha caipira e identificar as funções biológicas previstas usando as vias da Enciclopédia de Gene e Genômica de Kyoto (KEGG) e interação proteína-proteína. *Suabes cloacais* foram coletados de galinhas criadas ao ar livre em fazenda local na cidade de Duhok. Os isolados foram cultivados e o teste bioquímico realizado com XLD e TSI, respectivamente. A detecção molecular e anotação funcional do gene invA foi realizada usando PCR convencional e abordagens de bioinformática. O presente estudo descobriu que Salmonella foi detectada em 36 das 86 amostras usando métodos microbiológicos. Para confirmar esses achados, o gene invA foi utilizado e 9 dos 36 isolados de Salmonella mostraram um sinal positivo de invA pelo gel de agarose. Além disso, a análise bioinformática revelou que o gene invA estava associado principalmente a processos de secreção bacteriana, assim como seus termos KEGG e interação proteína-proteína estavam envolvidos na invasão bacteriana e nas vias de secreção. Esses achados sugeriram que o gene invA desempenha um papel importante na regulação dos processos de colonização e invasão de Salmonella dentro do hospedeiro intestinal na galinha caipira.

Palavras-chave: invA, Salmonella, galinha caipira, PCR convencional.

1. Introduction

Salmonella is a significant cause of poor health in human and animal worldwide. Studies indicate that more than 90 million cases of foodborne disease caused by Salmonella enterica subsp. enterica serovar Typhimurium. Food such as poultry, eggs and dairy products are considered as the major sources of Salmonella infections (Balasubramanian et al., 2019). The main symptom of Salmonella infection is gastroenteritis, however, it can cause typhoid fever, and paratyphoid fever or even cause death in serious infections in human (Eng et al., 2015).

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Salmonella enterica is a enteropathogenic a flagellated rod-shaped Gram-negative bacilli that is consider as key cause of enteric illness worldwide (Gal-Mor et al., 2014; Balasubramanian et al., 2019). The species S. enterica is divided into six subspecies: enterica, salamae, arizonae, diarizonae, houtenae, and indica and each of them has many serotypes or serovars (Kirk et al., 2015). Salmonella enterica serotype Typhimurium (S. Typhimurium) and Salmonella enterica serotype Enteritidis (S. Enteritidis) are the most common cause of disease in humans and veterinary animals in the world. Poultry and its products are the main sources of infection with Salmonella and can easily transmit salmonella from reservoirs to other animals and humans (Kirk et al., 2015; Besharati et al., 2020; Vaez et al., 2020). Poultry infected with S.pullorum and gallinarum reveals symptoms of white diarrhoea and fowl typhoid. Early detection of salmonella has been used to improve the poultry reproductive efficiency in order to meet a rapid rise in Global requirements for meat and egg products. However, this can be affected by several factors including the presence of known virulence factors that have been associated with severe human disease in salmonella species. Recently, free-range chicken farms have become major contributors to organic food production in several Asian countries including Iraq due to health and economic benefits (Rossi et al., 2018). The young chickens usually obtained from local commercial poultry hatchery or can be hatched under hen, afterward they raised without using antibiotics or growth hormones for therapy or enhancing growth compared with chickens raised in commercial broiler farms (Zhao et al., 2016). Free-range chickens in developing countries are very popular because they can easily access to green spaces, consume leftovers and less antibiotics usage. In addition, free range chicken can provide a side income and a good source of meat and eggs (Padhi, 2016). However, free range chicken grows very slowly and more likely prone to diseases including gastroenteritis that caused by Salmonella spp. (Jafari et al., 2007; Sibanda et al., 2020). Improvement in poultry production will be achieved by better understanding the molecular mechanism that underlying the Salmonella pathogenicity as a result of stimulation of the several virulence factors of the salmonella strains. Several groups have developed different molecular techniques and bioinformatic software to study the genome of Salmonella (Shi et al., 2015; Diep et al., 2019). Moreover, Studies have revealed that several invasion genes such as a subset of Inv genes can control the invasion and colonisation of Salmonella enterica and typhimurium in the gut wall of young aged chick and adult chicken (Harvey et al., 2011). In addition, several virulence genes have been involved in colonisation in the gut system of different species, including Salmonella pathogenicity islands (SPI1 and SPI2) (Ilyas et al., 2017). This study aimed to detect the salmonella isolated from the chicken cloaca using microbiological and molecular approaches as well as provide an insight into the functional roles of invA gene in invasion and colonisation of Salmonella spp.

2. Materials and Methods

2.1. Ethics statement

Ethical approval was given by the ethics committee at the college of Veterinary Medicine, university of Duhok (DR2020820CV) for all experimental procedures.

2.2. Sample collection

86 fresh cloacal swabs were obtained from three free-range chicken farms in Duhok city, Iraq. Fresh samples were collected using sterile cotton swabs. After sampling, the swabs were transported to Duhok research centre at the College of Veterinary Medicine, University of Duhok and processed for microbiological and molecular studies.

2.3. Isolation of Salmonella isolates

The cotton swabs were immediately put into vials containing 10 mL of warm buffered peptone water for non-selective pre-enrichment, shaken sufficiently and incubated overnight with swab at 37 °C. Then 0.1 mL of the enrichment broth was added to Rappaport-Vassiliadis Medium at 42 °C for 24 h. Afterward, A 10 µL bacteriological loop was used to streak the RVS culture on to Xylose Lysine Deoxycholate agar and incubated at 37 °C ± 1 overnight. Suspected Salmonella colonies were then sub-cultured to Xylose Lysine Deoxycholate (XLD) and incubated at 37 °C ± 1 for 24 h (Park et al., 2012). Suspected Salmonella isolates were maintained in 25% glycerol and brain heart infusion broth (BHIB) and stored at -20 °C for further processing.

2.4. Biochemical characterization of isolates

The Salmonella isolates were subjected to the Triple sugar iron (TSI) agar test. The test was performed according to the standard protocols. A pure colony was stab-inoculated into to the butt of the TSI medium and later streaked on the slant and incubated aerobically at 37 °C for 24 hours (Waltman et al., 2008). Salmonella-positive colonies were collected and kept under −20 °C for further use.

2.5. DNA extraction

Two to three colonies were collected from positive Salmonella Xylose Lysine Deoxycholate agar plates, placed in to microfuge tube and resuspended in 100 µL of sterile water. The suspension incubated at 100 °C for 10 min. The bacteria debris was removed by centrifugation (9600 g, 10 min) and supernatant was transferred into new tube, followed by incubating samples on ice for five minutes then stored at −20 °C until ready for use.

2.6. PCR characterisation of invA gene

A PCR was used to identify the isolates collected during sampling. Isolates were identified as Salmonella through the amplification of an InvA gene (primer pairs 5′-ACAGTGCTCGTTTACGACCTGAAT-3′ and 5′-AGACGACTGGTACTGATCGATAAT-3′) (Bhatta et al., 2007). The primers were blasted against the nucleotide database of the EBI (BLASTN, version 2.9.0+) website to ensure identity among reported BLAST sequences for

the target gene and the absence of significant homology with other microorganism sequences (EMBL-EBI, 2022). PCR reactions were conducted in a total volume of 20 µL. Each reaction contained 10 µL 2× Ruby PCR Master (Ruby Taq Master, Jena Bioscience. Germany), 0.3 µm of each forward and reverse primer for InvA and 2 µL DNA. PCR cycling conditions were as follows; 1 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30s at 60 °C and 2 min at 72 °C followed by 10 min at 72 °C (Chiu and Ou, 1996).

2.7. Gel electrophoresis of amplicons

Detection of invA amplicons was carried out using a 1.5% (w/v) agarose gel prepared from 1X tris acetate ethylenediamineacetate (TAE) buffer with Prime Safe Dye (GeNet Bio, Korea). A DNA marker (Jena bioscience, Germany) of was used. The gel was allowed to run at 85 volts, 300 amperes for 45 mins in 1X TAE buffer and was visualised under the UV transilluminator light.

2.8. Gene enrichment analysis

ClueGO (v2.5.7) software was used to detect Gene Ontology (GO) term and Kyoto Encyclopedia of Gene and Genome (KEGG) pathways. ClueGO enable analysis of genes from several organism including salmonella and it can recognise many identifier types based on information from different sources such as NCBI, UniPrtKB and Ensembl (Bindea et al., 2009). Two-sided hypergeometric test used for enrichment analyses, Benjamini–Hochberg correction was utilized for P value correction and Kappa coefficient of 0.4 was used to detect the similarities of GO terms for associated genes. The resulting GO terms with $P<0.01$ and KEGG pathways with $P<0.05$ were considered significant. Furthermore, the results were constructed and visualized by Cytoscape (v3.8.2)

2.9. Protein -protein interactions network

Protein-protein interaction analysis of the miRNA target genes was performed using STRING (2022) based on Salmonella species. STRING is an online tool designed to determine the potential predicted and experimental information of interaction between proteins. The interactions between the invA gene and associated proteins were mapped into PPIs network with confidence score of >0.7 as the cutoff standard. Cytoscape-plugin known as network analyzer (v4.4.6) was utilized to construct the score of gene nodes.

3. Results

3.1. Identification of Salmonella using microbiological methods

Out of 86 isolates, 36 were salmonella positive based on their characteristics of colony morphological and biochemical properties. Salmonella was identified by typical pink with black centers colonies on the XLD agar and can be easily differentiated from other organisms (Figure 1A). Confirmatory test was also carried out on the Salmonella spp. using TSI which measures the precipitation of hydrogen sulfide and utilization of glucose, lactose and sucrose. Salmonella isolates found to be TSI positive, the slant showed red color of the medium, the butt was yellow due to glucose fermentation as well as the TSI test revealed blackening of medium as a result of H2S production (Figure 1B).

3.2. Identification of Salmonella using conventional PCR

The goal of this study was to elucidate the detection of the InvA gene of Salmonella in the cloacal samples of the free-range chicken. The InvA primer used in this study was designated by Bhatta et al group is 244-bp long and located on chromosome I of Salmonella spp. Using EBI-BLASTN+, Blast alignment analysis revealed that this sequence was 100% similar with several Salmonella species such as Salmonella gallinarum, Salmonella enterica subsp. enterica serovar Typhimurium, paratyphi, enteritidis and pullorum (Table S1). PCR amplification of positive invA gene was observed by agarose gel (Figure 2). Among of the 36 representative isolates, about 9 isolates showed positive amplification of the invA gene while about 27 isolates were negative.

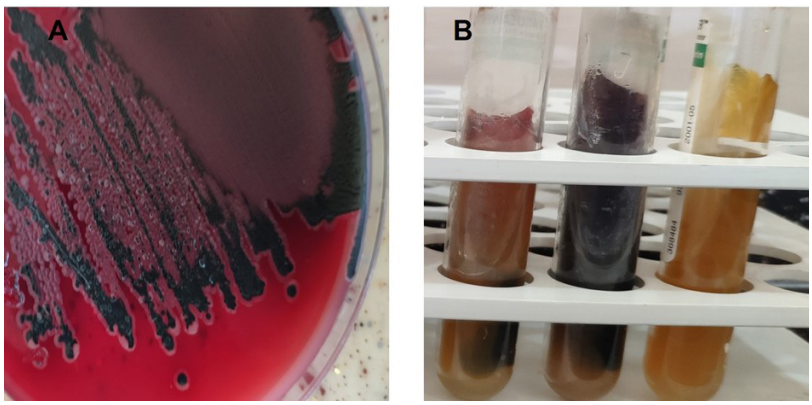

Figure 1. A representative schematic of the (A) Phenotypic characteristics of Salmonella spp. on the XLD agar; (B) TSI test showed positive production of H_2S and fermentation of glucose tube 1 positive (Alkaline/Acid, -Gas, + H2S), tube 2 positive (Alkaline/Acid, -Gas, + H2S), tube 3 negative (Acid/Acid).

3.3. Gene enrichment functional analysis

Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomics (KEGG) pathway analysis showed that invA was significantly enriched for 6 biological process (BP) and 3 cellular components (CC). The most significant GO terms were secretion by cell (GO:0032940) and plasma membrane (GO:0005886) in BP and CC categories, respectively (Figure 3). KEGG enrichment showed significantly enriched pathways for invA gene, including bacterial invasion and bacterial secretion pathways (sty05100, sty03070), respectively (Figures 4 and 5).

3.4. PPI network construction and network analysis of target genes

Protein interactions were explored and analyzed using STRING. As shown in Figure 6A, a total of 1635 edges and 91 nodes were obtained, Using the network analyzer in Cytoscape, we identified top core genes with the highest node degrees (spaO, invF, prgH, sipB, invE, invG, spaI, spaS, sptP, sspH, sicA, prgK, envE, spaP, spaN, sopB, sopD) (Figure 6B). These results showed a significant enrichment of genes for GO terms such as peptide secretion (GO:000279). Top KEGG pathways significantly associated with genes in the module were Bacterial invasion of epithelial cells (KEGG:05100) and Salmonella infection (KEGG:05132).

4. Discussion

Salmonella considers as major cause of food born disease in human and animals worldwide. Backyard or free-range chicken have been widely grown by individuals as a source of fresh nutritious eggs and meat, however, free-range chicken is more susceptible to transmit Salmonella and induce infection among species (Ghoddusi et al., 2015). The conventional microbiological methods, including culture using selective medium such as XLD and biochemical test such as TSI have shown that 36 out of 86 cloacal samples were identified to be Salmonella spp. However, due to low specificity of these techniques (Sousa and Pereira, 2013), these results were validated using invA gene as key marker for detection of Salmonella. invA gene is highly conserved across Salmonella species, located in Salmonella pathogenicity island-1 and it is critically involved in regulating pathogenicity and

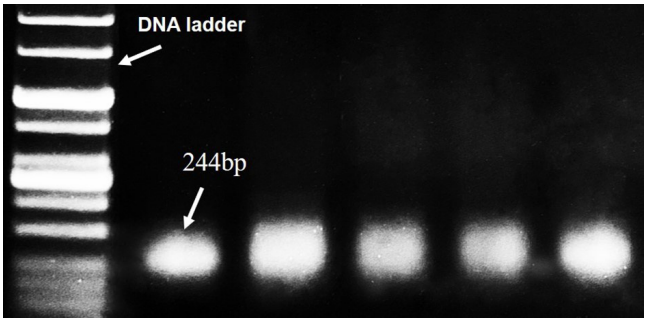

Figure 2. Analysis of PCR amplified invA gene on the 1.5% agarose gel electrophoresis. Lane 1 is a 100 bp DNA marker), other lanes (244 bp) are the amplified fragment for invA gene.

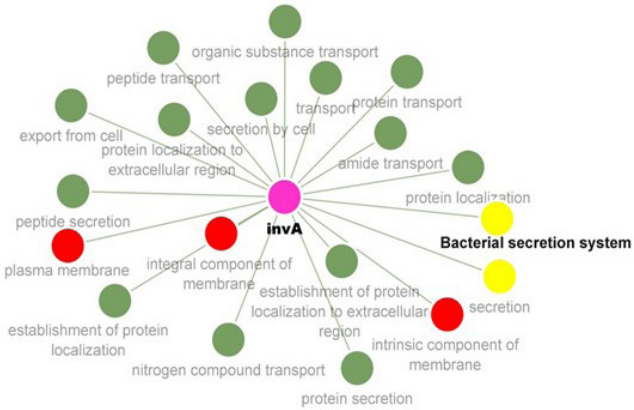

Figure 3. Functional GO terms and KEGG pathway enriched for invA gene using ClueGO. The node represents a GO term, each color represents a functional group and the node size represents the term enrichment significance, $p \leq 0.01$. A. Enriched biological processes (BP) (green), Cellular components (CC) (red colour) and KEGG pathway (Yellow).

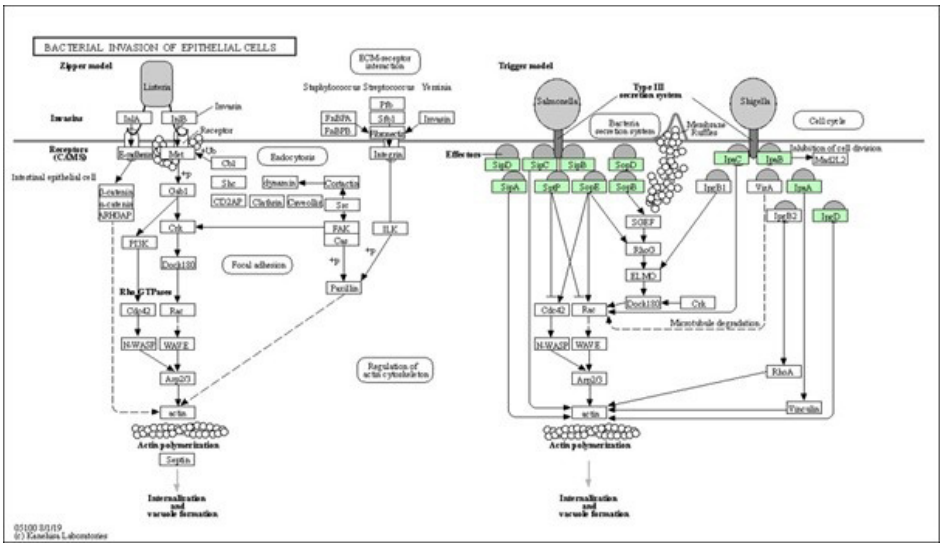

Figure 4. Bacterial invasion of epithelial intestinal cell pathway displayed as an interaction network using KEGG tool. Several pathways have been involved in regulation of invasion including the bacterial secretion system (GenomeNet, 2022a).

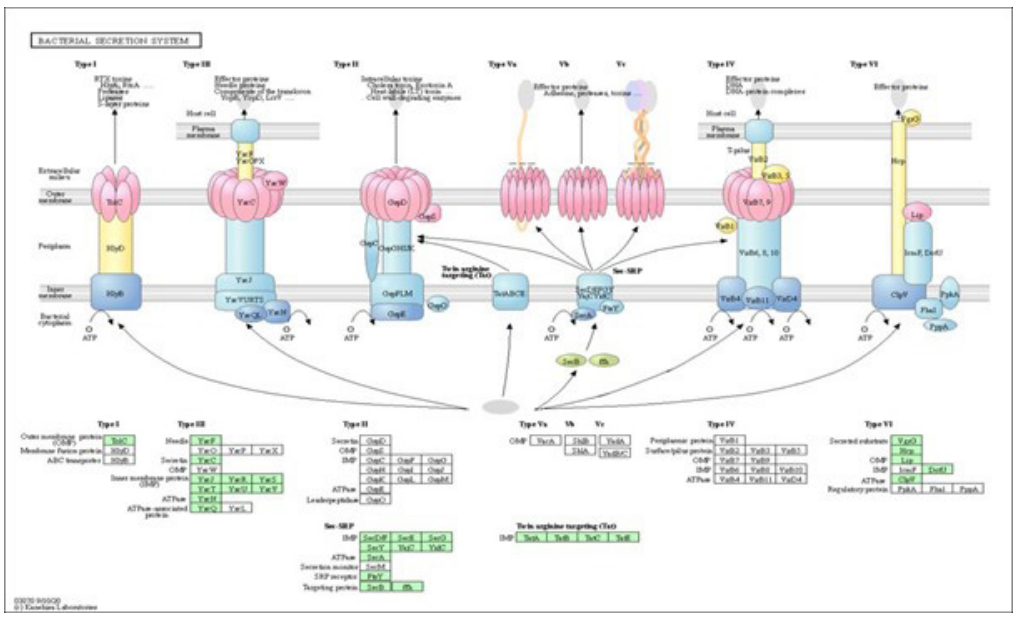

Figure 5. Bacterial secretion pathway displayed as an interaction network using KEGG tool (GenomeNet, 2022b).

invasion of salmonella in the host intestinal mucosa (Shanmugasamy et al., 2011). Using conventional PCR approach, 9 out of 36 of microbiologically Salmonella positive isolates were detected invA-positive in free range chicken which is in agreement with the results of previous studies suggested that PCR amplification of invA gene made a reliable rapid diagnosis of salmonella spp. in the samples compared with samples as evaluated by the traditional cultural and biochemical methods (El-Sharkawy et al., 2017; Siala et al., 2017) in pig, ruminant and chicken (Scholz et al., 2001; Al-Iedani et al., 2015; Akinola et al., 2019; Mkangara et al., 2020). In the present study, the overall rate of Salmonella isolates in the free-range chicken was significantly lower than that of the commercial raised chicken (Harvey et al., 2011; Jajere et al., 2019). Our bioinformatic results showed that invA gene was involved in pathogenicity of Salmonella and protein secretion which is mediated by the subset of genes, located on the chromosome or the plasmid (Guo et al., 2000; Ilyas et al., 2017). Moreover, several studies have showed that invA gene is a component of the type III secretion system and plays an essential role in mediating host recognition, leading to induction of invasion in the epithelial cells of intestinal mucosa, subsequently caused colonization in these cells, and causes enteritis disease (Darwin and Miller, 1999). Interestingly, the invA gene

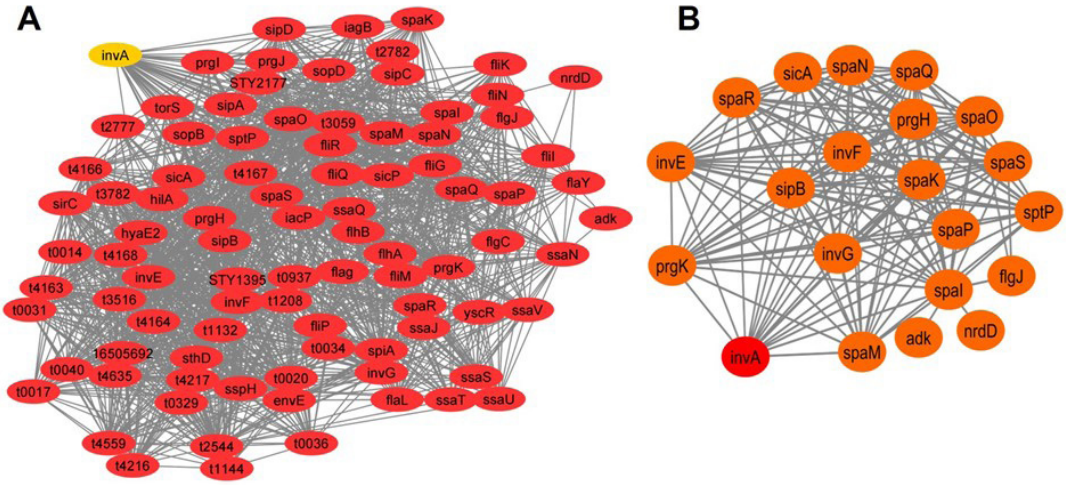

Figure 6. (A) PPI networks show the interaction of invA with the associated proteins. The nodes and edges are constructed using the STRING tool and visualized by Cytoscape software. The nodes represent the genes, while edges represent the protein- protein associations; (B) Top significant subnetwork module was identified from the PPI network using the network analyzer with highest score.

was significantly enriched in cellular component related to plasma membrane and the top significant KEGG term enriched for invA gene was bacterial invasion of the epithelial cell. The genes in the bacterial invasion pathway appears to be associated with several signalling pathways such as bacterial secretion system in the several species of Salmonella (Kim et al., 2018). The invA gene is one of the several virulence genes that form a cluster associated with host invasion and bacterial secretion properties. In the present study, large number of virulence genes identified through PPI analyses were significantly associated with Salmonella pathogenicity island 1 (SPI-1)-encoded type III secretion system (T3SS1) components (Porta et al., 2014) . Studies revealed that deletion of invA, ssaK, and flgB can be detrimental to the bacteria as it can block T3SS1, T3SS2, and flagellar apparatus, respectively. InvA forms the export apparatus required for the assembly of functional needle complex of T3SS1 and mutants lacking InvA resulted in loss of function to translocate T3SS1 effectors which in turn suppressed the secretion of SipC and SopE2 through T3SS1 (Collazo and Galán, 1997; Monjarás Feria et al., 2015; Kim et al., 2018). Moreover, Salmonella-induced membrane ruffling in intestinal epithelial cells was enhanced by four T3SS1 effectors associated with OMVs; SopB and SopE2, SipA and SipC that are responsible for rearrangement of actin filaments (Suárez and Rüssmann, 1998; Raffatellu et al., 2005; Kim et al., 2018). Other T3SS-related proteins including the cytoplasmic proteins OrgA, OrgB and InvC, as well as the protein translocases SipB, SipC and SipD. OrgA and OrgB, SpaO were identified as components of the needle complex and export apparatus of Salmonella enterica that play a pivotal role in the invasion of bacteria within the host cells (Lara-Tejero and Galán, 2009; Lara-Tejero et al., 2011, 2019). The spaN gene is also needed by Salmonella to facilitate its entry into non-phagocytic cells and destruction of macrophages. Moreover, InvA, SpaP, SpaQ, SpaR, SpaS, Inv, PrgH, PrgK and PrgI proteins form the needle complex of the export apparatus that plays a role in the intracellular invasion and survival of Salmonella within the host as well as it is also linked to systemic Salmonella infections (Cornelis, 2006; Galán and Wolf-Watz, 2006; Worrall et al., 2010; Monjarás Feria et al., 2015; Lou et al., 2019). In addition, knock out of Salmonella typhimurium hilA gene is associated with colonization resulted in the reduction of expression of invA and invF genes which validate the essential role of hilA in the regulation and activation of the expression of invasion genes (Safarpour-Dehkordi et al., 2018). These biological processes and pathways reflect the critical roles of invA as key mediator of bacterial invasion as well as colonization in the intestinal epithelial cells of the infected animals and human.

5. Conclusion

Overall, this study demonstrated that presence of invA in the Salmonella isolates of the free-range chicken, provided obvious evidence of potential role of invA as biomarker for detection of Salmonella in free range poultry which was more efficient than using traditional microbiological methods. Bioinformatics analysis suggested an involvement of invA gene in Salmonella secretion system, invasion and pathogenesis. Finally, this information may assist to design new therapies to treat Salmonellosis in animal and human through inhibition of the action of the invasion genes including invA.

Abbreviations

GO Gene ontology
KEGG Kyoto Encyclopedia of Gene and Genomic
BP Biological process
CC Cellular components

invA, invF, invE, invG Invasion proteins
sipB Cell invasion protein SipB
sptP protein tyrosine phosphatase
sspH Salmonella secreted protein H1
sicA Salmonella invasin chaperone
prgK, prgH Lipoprotein Prg
envE Probable lipoprotein EnvE
spaP, spaN,spaO, spaI, spaS Surface presentation of antigens protein
hilA hyperinvasive locus A
sopB, sopD secreted protein in the Sop family
spp Species
TSI Triple sugar iron
XLD Xylose Lysine Deoxycholate
RV Rappaport-Vassiliadis Medium

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. The invA primer was blasted against the nucleotide database of the EBI (BLASTN, version 2.9.0+). This material is available as part of the online article from https://doi.org/10.1590/1519-6984.263363