Sequencing of bcfC Gene of _Salmonella Typhimurium_ Isolated from Ducks in Egypt

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ABSTRACT

The main objective of this study was to applying bcfC gene sequence of _Salmonella Typhimurium_ recently isolated from ducks to give insight into the source and origin, molecular epidemiology, disease pattern of _Salmonella Typhimurium_ in Egyptian duck farms. Out of 75 fecal swab samples, 15 (20%) local field isolates were detected and confirmed phenotypically by culturing, gram staining, biochemically and serologically to be _Salmonella Typhimurium_. The PCR amplification with bcfC gene-specific primers was conducted with genomic DNA, which revealed a product with the approximate size of 467 bp. The bcfC gene was found in 7 (46.6%) isolates of _Salmonella Typhimurium_. Phylogenetic and partial gene sequence analysis of bcfC gene of _Salmonella Typhimurium_ showed clear clustering of Egyptian isolates of _Salmonella Typhimurium_ and different _Salmonella_ strains uploaded from GenBank. Sequence identities between the isolated Egyptian strain and different _Salmonella Typhimurium_ strains from GenBank revealed 99.8-100% homology. Open reading frame (ORF) analysis of _Salmonella Typhimurium_ bcfC gene using NCBI tool and ORF analysis of bcfC gene protein translation using ExPasy (SIB Bioinformatics Resource Portal) indicated all open reading frames of a specified minimum size in a sequence of (453 bp). The 3 conserved domains region in the nucleotide sequence were PapC N-terminal domain (107-394bp), PRK15193 outer membrane usher protein (56-424bp), and FimD Outer membrane usher protein FimD/PapC (cell motility, extracellular structures, 56-424bp). The PapC N-terminal domain was a structural domain found at the N-terminus of _S. typhimurium_ PapC protein and had a central role in the pili assembly chaperone usher system (CUP). Amino acids alignment report of the sequenced 415 amino acid of _Salmonella Typhimurium_ bcfC gene showed great homology between the Egyptian _Salmonella Typhimurium_ strain and the different _Salmonella_ strains uploaded from GenBank. Nucleotide alignment report of the sequenced _Salmonella Typhimurium_ bcfC gene at (417bp) demonstrated great homology between the Egyptian _Salmonella Typhimurium_ strain and the different _Salmonella_ strains uploaded from GenBank. In conclusion, the Egyptian _Salmonella Typhimurium_ isolate was related to the common sequence types isolated from humans and bovine-based products across the world especially in the United Kingdom, USA, Ireland, and México. Most of the duck farms from which we isolated the Egyptian _Salmonella Typhimurium_ isolates were located in the same geographical area of cattle farms in addition to the duck farms lacked the requirements of biosecurity, which could facilitate the circulatory transmission of salmonella strains between the human beings and other animal farms, including duck farms. Moreover, the PapC N-terminal domain was a central conserved domain encoded by bcfC gene of _S. Typhimurium_. A PapC N-terminal conserved domain can be used as a vaccine target for vaccine production against _S. Typhimurium_.

Keywords: bcfC gene, Conserved domain, Duck, GenBank, ORF, Phylogenetic tree, _Salmonella Typhimurium_, Sequencing.

INTRODUCTION

_Salmonella_ infections are considered one of among the foremost major problems within the poultry industry. _Salmonella Typhimurium_ has been regarded to be frequently related to disease in numerous species, including humans, livestock, domestic fowl, rodents, and birds. Therefore, _Salmonella Typhimurium_ is described as the prototypical broad-host-range serotype (Rabsch et al., 2002). _Salmonella typhimurium_ has been found in 60% of poultry carcass (Mann and McNabb, 1984) and is responsible for 93% of the _Salmonella_ infections in ducklings (Badr and Nasef, 2016). _Salmonella Typhimurium_ has been isolated from 40% of hatchlings and 1% of older ducklings in Taiwan, although clear host species specific differences have also been detected. 12 Salmonella has been previously isolated from imported day old ducklings in Brazil and also the USA (Ribeiro et al., 2006 and Gaffga et al., 2012). Because the prevalence of Salmonella in duck products poses a risk to human populations, an urgent need exists to research the prevalence, disease risk to human populations, and also the global epidemiology of _Salmonella_ serovars and specific clones. This information could also be wont to address Salmonella risk and promote evidence-based interventions in global public health (Osman et al., 2014).
Pili (fimbriae) play a central role in bacterial colonization and pathogenesis (Li and Thanassi, 2009). Fimbriae are proteinaceous extracellular structures and play a distinct role in adhesion, a major initial step for colonization and entry into host cells. Fimbriae have also referred to as to play a central role in interactions with macrophages, intestinal persistence, biofilm formation and bacterial aggregation in Salmonella serovars (Ledebroer et al., 2006). The fimbral gene (bcfC) is located on a fimbral structure and play a vital role in attachment and cell invasion of Salmonella typhimurium (Huehn et al., 2010). Fimbrial gene bcfC is widely distributed among Salmonella, these data are according to the essential functions of adhesion factors for the attachment and internalization processes that occur during pathogenesis (Borriello et al., 2012).

bcfC is fimbral usher protein consists of three functional domains which are PapC N-terminal domain, PRK15193 outer membrane usher protein and FimD outer membrane usher protein FimD/PapC. PapC (pyelonephritis-associated pilus C) is an integral outer membrane usher protein that forms an assembly platform for pilus biogenesis, PapC has five functional domains, all of which are required for pilus biogenesis, It's a 24-stranded β-barrel transmembrane domain that permits translocation of the polymerized pilus fiber across the outer membrane and 4 globular domains: a periplasmic N-terminal domain (NTD), two periplasmic C-terminal domains (CTD1 and CTD2), and a plug domain (Plug) (Henderson et al., 2011 and Phan et al., 2011). The usher PapC N-terminal functional unit represents primary binding site for chaperone-usher formation (Ng et al., 2004; Nishiyama et al., 2005 and Li et al., 2010).

Therefore, the main aim of this study is applying genetic sequencing and phylogenetic analysis of bcfC gene by using bioinformatics approach to explore information about bcfC protein and to give insight about the source and origin, molecular epidemiology, disease pattern of Salmonella Typhimurium in Egyptian duck farms. Also, identification of highly conserved domains in Salmonella Typhimurium bcfC gene sequences for vaccine designing production against Salmonella typhimurium.

MATERIALS AND METHODS

Ethical approval

No ethical approval was obtained from the Institutional Animal Ethics Committee because no invasive procedure was performed on the animals. However, this study was conducted in accordance to the Institutional Animal Ethics of Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo, Agricultural Research Center (ARC).

Samples collection

Totally, 75 fecal swabs were collected that contained 30 from apparently healthy ducks and 45 from diseased ducks, these were collected from five duck farms in Qaluobia, Sharkia and Monofia governorates of Egypt.

Isolation of Salmonella Typhimurium

It was carried out according to methods described by ISO6579 (2002)

Identification of Salmonella Typhimurium

Microscopic examination

Suspected colonies were Gram's stained and microscopically examined according to methods described by Quinn et al. (2002).

Biochemical identification

Biochemical identification was performed on isolated organisms by using the Analytical Profile Index 20E (API 20E) system (Nucera et al., 2006).

Serological identification

Salmonella culture serotyping was carried out according to methods described by Kauffmann-White typing scheme (Popoff, 2001).

Molecular identification

DNA was extracted using the QIA amp DNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer’s instructions with modifications. PCR was performed on extracted DNA by using specific primer (Table 1) supplied from Metabion (Germany) to amplify bcfC gene according to Huehn et al. (2010). PCR was performed in a 25μl reaction containing 12.5 μl of Emerald Amp GT PCR Master Mix (Takara, Japan), 1 μl of each primer of 20 p/mol concentrations, 4.5 μl of water and 6 μl of DNA template, using an Applied Biosystems 2720 Thermal Cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 μl of the products was loaded in each gel slot. A100 base pair (bp) DNA Ladder (Qiagen, Germany, GmbH) was utilized to determine the fragment sizes. The gel was photographed by means of a gel documentation system (Alpha Innotech, Biometra).
Surface organelles, play an important role in the infection of eukaryotic pathogens. DNA sequences were obtained by PCR amplification of Salmonella Typhimurium (453 bp). ExPASY-Translate Tool-SIB Bioinformatics Resource Portal was used for ORF analysis of bcfC gene sequence of Salmonella Typhimurium (453 bp) (https://web.expasy.org/translate/).

**Conserved domain Search**

NCBI Search Tool was performed conserved domain analysis of the bcfC protein sequence.

**Phylogenetic, amino acids and nucleotide sequence analysis of bcfC gene of Salmonella Typhimurium**

It was performed in Elim biopharmaceuticals, Germany. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis was initially performed according to standard methods described by Altschul et al. (1990). A comparative analysis of nucleotide and deduced amino acids sequences was performed using the CLUSTALW multiple sequence alignment program, version 1.83 of Mega Align module of Lasergene DNA Star software in accordance to methods designed by Thompson et al. (1994) and phylogenetic analysis was performed using neighbor joining in MEGA6 (Tamura et al., 2013).

**RESULTS AND DISCUSSION**

**Isolation and identification of Salmonella Typhimurium field isolates**

Out of 75 fecal swab samples, 15 isolates were confirmed phenotypically, biochemically and serologically to be Salmonella Typhimurium in a prevalence of 20% (15/75). These finding agree with Osman et al. (2014) (18.5%) and disagree with Abd El-Tawab et al. (2015) (9.6%), Lebdah et al. (2017) (14.2%), Hoszowski and Wasyl (2005) (14.3%) and Ismail (2013) (27.02%). The PCR amplification of Salmonella Typhimurium bcfC gene using specific primer sequences revealed an approximate size of 467 bp (Figure 1). bcfC gene was found in (7/15) (46.6%) Salmonella Typhimurium isolates. These results disagree with Osman et al. (2014) (100%) and Lebdah et al., (2017) (100%).

In this study, the ORF Finder commonly used on the NCBI tools website was performed (Figure 2). The NCBI tool determines the using of all qualified and alternative genetic codes. The ORF finder locates all ORFs of a specified minimum size during a nucleotide sequence. The sequence when subjected to ORF finder showed that all ORFs found of Salmonella Typhimurium (453 bp) (Figures 2 and 3). Also the ORF 2 is the largest one (348 nt) and it begins at complement (2-349 nt). It encodes 115 amino acids (aa). While ORF 1 is found downstream to ORF 2 is about (246 nt) long from (207-452 nt). It encodes 81 aa. ORF finder gives information about the coding and non-coding sequences. Detecting the coding and non-coding regions and final product in the form of its amino acid sequences is essential for understanding the evolutionary processes in various pathogens. By analyzing the ORF we will predict the possible amino acids that are producing during the translation process. The prediction of the proper ORF from a newly sequenced gene is a vital step. ORF is essential to design the primers which are required for PCR and sequencing (Orr et al., 2019).

Nucleotide sequence (453bp) was used to predict the domain region in the sequence (Figure 4). PapC N-terminal domain (107-394bp), PRK15193 outer membrane usher protein (56-424bp) and FimD outer membrane usher protein (56-424bp). Three domains were found in the bcfC gene sequence of Salmonella. The PapC N-terminal domain is a structural domain found at the N-terminus of the Gram-negative bacteria PapC protein. Pili are assembled using the chaperone usher system. In Gram-negative bacteria, this is can be composed of the chaperone PapD and the usher PapC, this domain constituent the N-terminal domain from PapC. N-terminal domain have a central role in substrate binding. The fimbrial usher protein is play a central role in assembling of the pilus in Gram-negative bacteria. Pili known as one of the major fibrous surface organelles, play a great role in attachment to host tissues and given rise to development of a variety of diseases (Nougayrede et al., 2003). The assembly of fimbrae (or pili) need 2 components for assembly and transport system which consist of a periplasmic chaperone and an outer 'usher' membrane protein (Saier and Rosmalen 1993; Hultgren et al., 1994; Schifferli and Alrutz, 1994). The usher protein has a molecular weight of 86-100 kDa and include a membrane-spanning 24-stranded beta barrel domain, reminiscent of porins, and of 4 periplasmic soluble domains: an N-terminal one of about 120 residues (NTD) (Nishiyama et al., 2005; Huang et al., 2009), a 'middle' domain (plug domain) about 80 residues long (Capitanli et al., 2006) and two

**Table 1. Primer sequences, target gene and amplicon size**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>Amplified segment product (base pair)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>bcfC</td>
<td>F-5'-accagagacattgcccct c-3’</td>
<td>467</td>
<td>Huehn et al., (2010)</td>
</tr>
</tbody>
</table>

**Open reading frame analysis**

The NCBI tools website was carried out for Open reading frame analysis (ORF) analysis of bcfC gene sequence of *Salmonella Typhimurium* (453 bp). ExPASY-Translate Tool-SIB Bioinformatics Resource Portal was used for ORF analysis of bcfC gene sequence of *Salmonella Typhimurium* (453 bp) (https://web.expasy.org/translate/).
IG-like domains (each about 80 residues long) at the C-terminus (CTD1 and CTD2 (Phan et al., 2011). Interaction between the NTD and Plug domains is essential step for usher gating. A conserved and immunogenic domain considered as a unique target for various vaccine development against Salmonella (Jha et al., 2015; Singh et al., 2017).

Phylogenetic and partial gene sequence analysis of bcfC gene of Salmonella Typhimurium that was generated using neighbor joining in MEGA6 (Figure 6), showed three major clusters or branches, one representing the Egyptian Salmonella Typhimurium strain isolate with CP022491.1, LT795114.1, CPO22497.1, LT571437.1, CPO14975.1, CPO14358.1, CPO22658.1, CPO18657.1, CPO24619.1, LN999997.1, CPO14356.1, CPO11233.1, CPO14977.1. the second cluster for CPO16754.1, CPO22003.1, CPO18659.1, CPO18655.1, CPO18635.1, CPO17232.1, CPO19633.1, CPO19383.1, CPO15526.1, CPO15524.1, CPO18661.1, CPO18651.1, CPO18648.1, and the third one for AF129435.1 and AF130422.1.

Nucleotide sequence distance of Salmonella Typhimurium bcfC virulence gene (figure 7) was created by the Mega Align module of laser gene DNA star. Sequence identities between the isolated Egyptian strain and different Salmonella Typhimurium strains uploaded from GenBank revealed that 99.8% to 100% homology. Nucleotide sequence analysis of bcfC virulence gene of the Egyptian isolated strain showed 100% nucleotide identity with the American Salmonella enterica subsp. enterica serovar Typhimurium strain CDC 2009K-1640 (accession No.CP014975), the American Salmonella enterica subsp. enterica serovar Typhimurium strain USDA-ARS-USMARC-1896 (accession No.CP014977) by Nguyen et al. (2016), the Irish Salmonella enterica subsp. enterica serovar Typhimurium strain SL1344RX (accession No.CP011233) by Fitzgerald et al. (2015), the Mexican Salmonella enterica subsp. enterica serovar Typhimurium strain YU15 (accession No.CP014358) and Salmonella enterica subsp. enterica serovar Typhimurium strain YU15-SO2 (accession No.CP014356) by Silva et al. (2016).

In this study the Egyptian Salmonella Typhimurium isolate was distributed into common sequence types isolated from humans and bovine-based products across the world especially in the United Kingdom, USA, Ireland and México. Most of the duck farms from which we isolated the Egyptian Salmonella Typhimurium isolates were located in the same geographical area of cattle farms in addition to these farms lacked the requirements of biosecurity, which facilitates the circulatory transmission generated between contaminated poultry meat and human beings.

So, this study concluded that it is possible that the realistic explanation for the existence of similar strains of salmonella typhimurium isolated from duck farms and cows farms and strains isolated from the human host is the absence of disinfection, sterilization operations and the absence of health requirements by farm workers in addition to that the duck farms were located in the same geographical area of bovine farms. Amino acids alignment report of the sequenced 415 amino acid of Salmonella Typhimurium bcfC gene showed (figure 5) great homology between the Egyptian strain and the different Salmonellae strains from GenBank. On the other hand, nucleotide alignment report of the sequenced 417bp of Salmonella Typhimurium bcfC gene showed (figure 8) high identity between the Egyptian strain and the different Salmonellae strains from GenBank.

**Figure 1.** Agarose gel showingPCR-amplified product of bcfC virulence gene of Salmonella Typhimurium isolated from ducks.Lanes (1, 4, 8, 9, 10, 12, and 14): samples positive for bcfC Gene (467 bp), Lane (pos.): positive control, Lane (Neg.): Negative control, Lane (L): MW 100bp ladder (DNA marker).
**Figure 2.** Open reading frame analysis of *bcfC* gene nucleotide sequence

**Figure 3.** Open reading frame analysis of *bcfC* gene protein translation using ExPasy (SIB Bioinformatics Resource Portal) showed all ORFs. The frame 2 is the longest one.

**Figure 4.** Conserved domains exist within the family region
Figure 5. Amino acids alignment of bcfc virulence gene of Egyptian isolated strain *Salmonella* Typhimurium and different *Salmonella* Typhimurium strains retrieved from GenBank using CLUSTALW multiple sequence alignment program version 1.83 of MegAlign module of Lasergene DNASTAR.

Figure 6. Phylogenetic tree for *Salmonella* Typhimurium bcfc virulence gene partial nucleotide sequences that was generated using neighbor joining in MEGA6, showing clear clustering of the Egyptian isolated strain (marked with red color) and different *Salmonella* Typhimurium strains uploaded from GenBank.
Figure 7. Nucleotide sequence distance analysis of *bcfC* virulence gene of Egyptian isolated strain and other *Salmonella* Typhimurium strains from GenBank.
**Figure 8.** Nucleotides alignment of bcfC virulence gene sequence of Egyptian strain of *Salmonella* Typhimurium and different *Salmonella* Typhimurium strains retrieved from GenBank using CLUSTALW multiple sequence alignment program version 1.83 of MegAlign module of Lasergene DNASTAR.

**DECLARATIONS**

**Author’s contributions**

Abeer Saad El-Maghraby designed the idea and concept of the review article, planned the study and Abeer Saad El-Maghraby, Abeer Mwafy and Hala Ahmed Al-Sawy designed and performed study design. All authors shared in writing, and approved the final version of manuscript.

**Acknowledgements**

This study was supported by the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Cairo and Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.

**Competing interests**

The authors declared that no competing interests exist.

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