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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).

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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 (Ledeboer et al., 2006). The fimbrial gene (*bcfC*) is located on a fimbrial 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 fimbrial 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 Nterminal 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 more 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).

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Table 1. Primer sequences, target gene and amplicon size

Microorganism	Gene	Primer sequences (5'-3')	Amplified segment product (base pair)	Reference
***Salmonella* Typhimurium**	*bcfC*	F-5'-accagagacattgccttc c-3' R-5'-ttctgatcgccgctattc g-3'	467	Huehn et al., (2010)

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/).

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 (453 bp) in (Figures 2 and 3). Also the ORF 2 is the largest one (348) nucleotides (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 FimD/PapC (cell motility, extracellular structures); (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 constituet the N-terminal domain from PapC. N-terminal domain have a central role in substrate binding. The fimbrial usher protein is play a centeral role in assembling of the pilus in Gram-negative bacteria. Pilus 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 fimbriae (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* (Capitani et al., 2006) and two

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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 CPO22491.1, 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, CPO18633.1, 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 enteric 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 of *Salmonella* strains between human and animal farms to duck farms, present results agree with Murgia et al. (2015); Ktari et al. (2016) and Yang et al. (2019). Wang et al. (2020) recorded that the relatively high frequency of invasive infection of *Salmonella* in commercial meat-type duck flocks may largely relate to semi-open rearing systems that lack effective biosecurity, the majority of *S.* Typhimurium isolates were grouped into ST19 (63.89%), the most common sequence types isolated from humans and animal-based food products across the world , Perhaps 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**.**

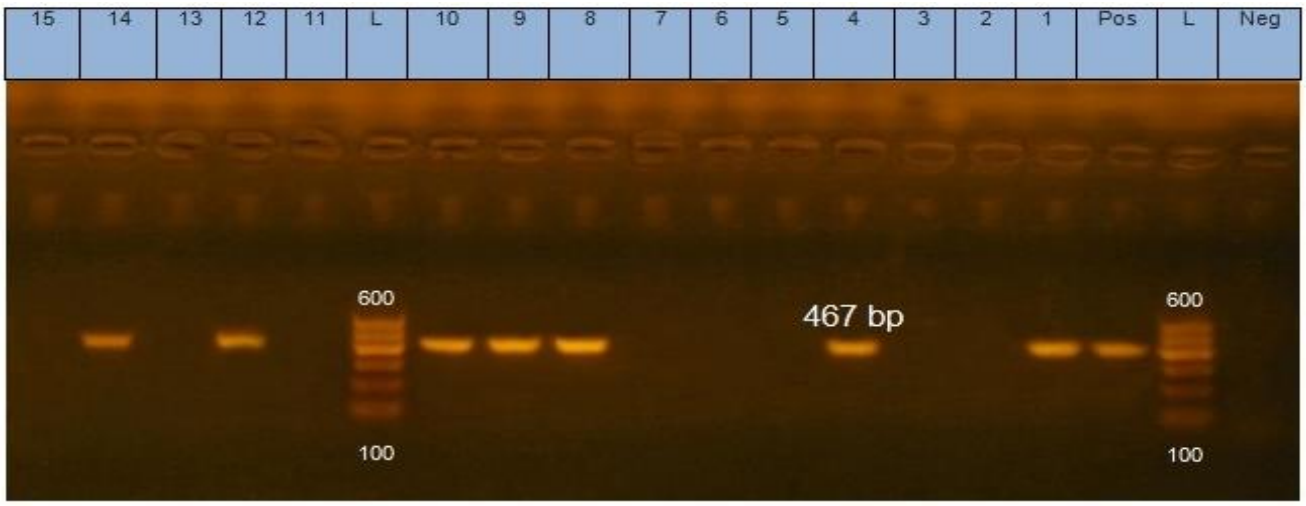

Figure 1. Agarose gel showingPCR-amplified product of *bcfC* virulence gene of *Salmonella* Typhimurium isolated from ducks.Lanes (1, 4, 8, 9, 10, 12, and 14): samples positive for*bcfC*gene (467 bp), Lane (pos.): positive control, Lane (Neg.): Negative control, Lane (L): MW 100bp ladder (DNA marker).

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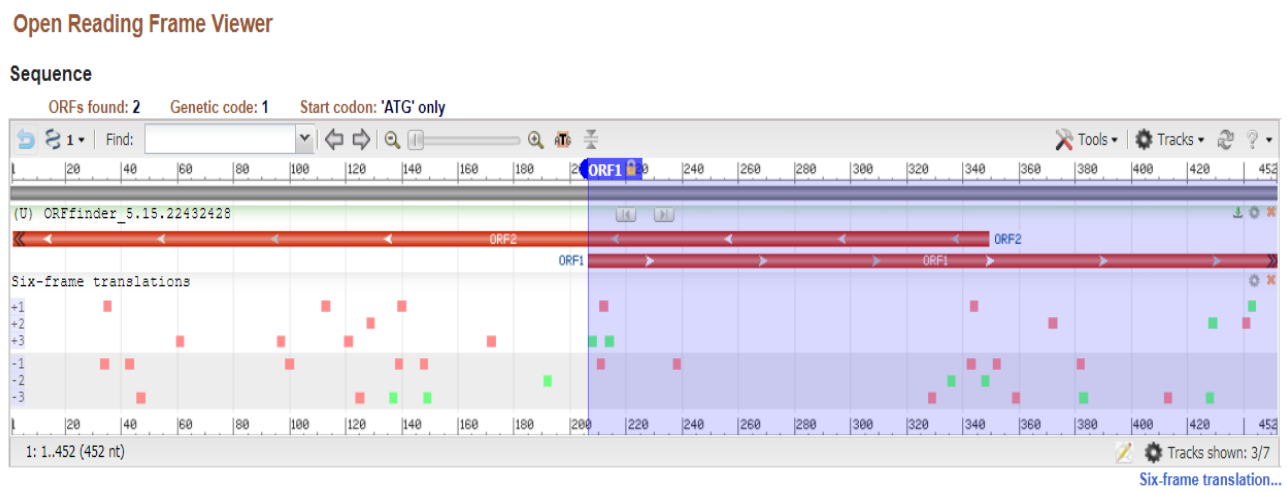

Figure 2. Open reading frame analysis of *bcfC* gene nucleotide sequence

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.

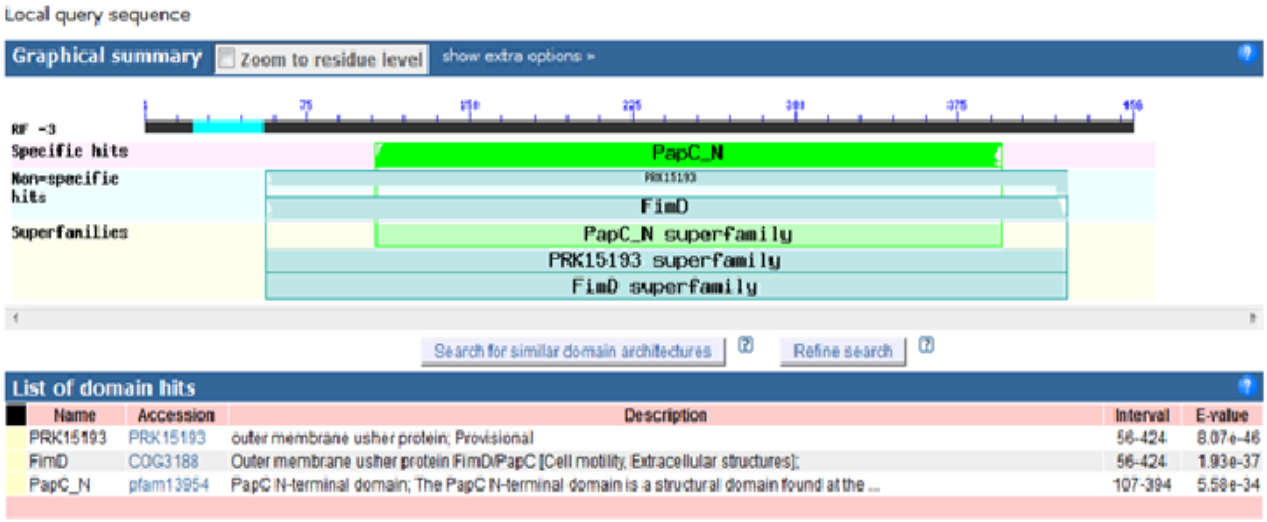

Figure 4. Conserved domains exist within the family region

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Majority   SPLFAFHVE-SRNRQAPRLRPLFNPRK-LFSALPPGLRS-LFAPVKV-FNSNALIPSSQYFGGI-PLALATIAACGMLMS
                    10        20        30        40        50        60        70        80
LT795114.1 ..................................................................................  238
Egy_strain ..................................................................................  238
CP022497.1 ..................................................................................  238
CP022491.1 ..................................................................................  238
LT571437.1 ..................................................................................  238
CP014977.1 ..................................................................................  238
CP014975.1 ..................................................................................  238
CP011233.1 ..................................................................................  238
CP014358.1 ..................................................................................  238
CP014356.1 ..................................................................................  238
LN999997.1 ..................................................................................  238
CP019383.1 ..................................................................................  238
CP015526.1 ..................................................................................  238
CP015524.1 ..................................................................................  238
CP018661.1 ..................................................................................  238
CP018651.1 ..................................................................................  238
CP018648.1 ..................................................................................  238
CP016754.1 ..................................................................................  238
CP022003.1 ..................................................................................  238
CP018659.1 ..................................................................................  238
CP018655.1 ..................................................................................  238
CP018635.1 ..................................................................................  238
CP018633.1 ..................................................................................  238
CP017232.1 ..................................................................................  238
AF129435.1 ..........G.....................................................................  238
AF130422.1 ..........G.....................................................................  238
CP024619.1 ..................................................................................  238
CP018657.1 ..................................................................................  238
CP022658.1 ..................................................................................  238

Majority   RRCCAKSKRVRAAGIALARSGQLPAAAFANSGNAPVFTPIFLSISGVRQARNPLSSVGA
                    90        100       110       120       130
LT795114.1 ..................................................................  415
Egy_strain ..................................................................  415
CP022497.1 ..................................................................  415
CP022491.1 ..................................................................  415
LT571437.1 ..................................................................  415
CP014977.1 ..................................................................  415
CP014975.1 ..................................................................  415
CP011233.1 ..................................................................  415
CP014358.1 ..................................................................  415
CP014356.1 ..................................................................  415
LN999997.1 ..................................................................  415
CP019383.1 ..................................................................  415
CP015526.1 ..................................................................  415
CP015524.1 ..................................................................  415
CP018661.1 ..................................................................  415
CP018651.1 ..................................................................  415
CP018648.1 ..................................................................  415
CP016754.1 ..................................................................  415
CP022003.1 ..................................................................  415
CP018659.1 ..................................................................  415
CP018655.1 ..................................................................  415
CP018635.1 ..................................................................  415
CP018633.1 ..................................................................  415
CP017232.1 ..................................................................  415
AF129435.1 ..................................................................  415
AF130422.1 ..................................................................  415
CP024619.1 ..................................................................  415
CP018657.1 ..................................................................  415
CP022658.1 ..................................................................  415
```

Figure 5. Amino acids alignment of *bcfC* virulence geneof 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.

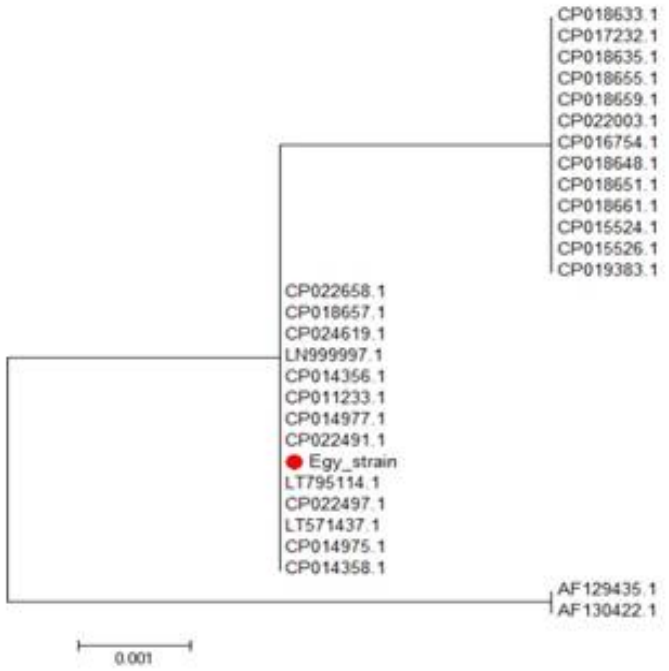

Figure 6. Phylogenetic tree for *Salmonella* Typhimurium *bcfC* virulence gene partial nucleotide sequences that was generated using neighbor joining in MEGA6, showing clear clustering of the Egyptian isolated strain (marked with red color) and different *Salmonella* Typhimurium strains uploaded from GenBank**.**

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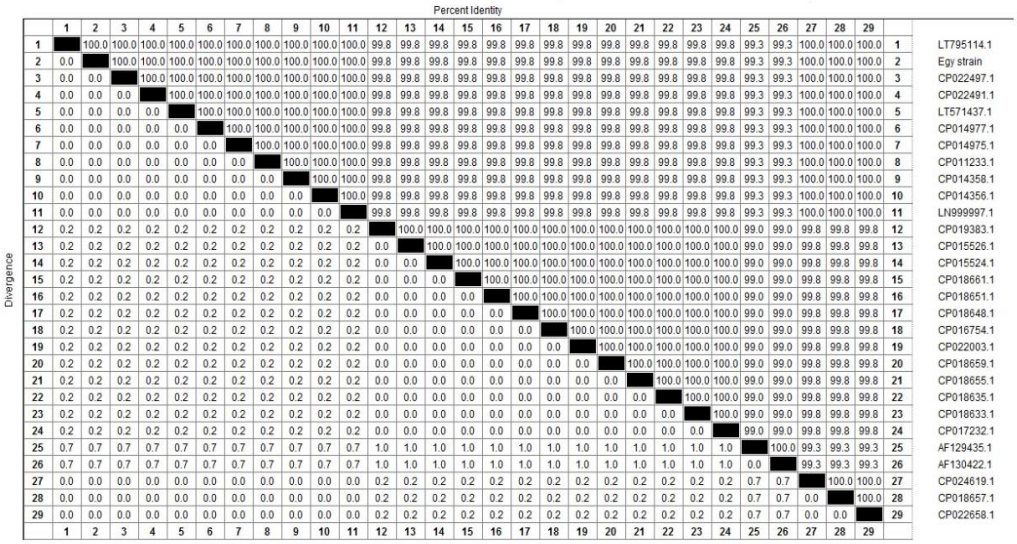

Figure 7. Nucleotide sequence distance analysis of *bcfC* virulence gene of Egyptian isolated strain and other *Salmonella* Typhimurium strains from GenBank.

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Majority   TCGCCGCTATTCGCGTTCCATGTGGAGTAGTCGCGTAACCGCCAGGCCCCCAGATTAAGGCCGCTATTCAATCCAAGAAA
                    10        20        30        40        50        60        70        80
LT795114.1 ..............................................................................  80
Egy strain ..............................................................................  80
CP022497.1 ..............................................................................  80
CP022491.1 ..............................................................................  80
LT571437.1 ..............................................................................  80
CP014977.1 ..............................................................................  80
CP014975.1 ..............................................................................  80
CP011233.1 ..............................................................................  80
CP014358.1 ..............................................................................  80
CP014356.1 ..............................................................................  80
LN999997.1 ..............................................................................  80
CP019383.1 ..............................................................................  80
CP015526.1 ..............................................................................  80
CP015524.1 ..............................................................................  80
CP018661.1 ..............................................................................  80
CP018651.1 ..............................................................................  80
CP018648.1 ..............................................................................  80
CP016754.1 ..............................................................................  80
CP022003.1 ..............................................................................  80
CP018659.1 ..............................................................................  80
CP018655.1 ..............................................................................  80
CP018635.1 ..............................................................................  80
CP018633.1 ..............................................................................  80
CP017232.1 ..............................................................................  80
AF129435.1 ..............................G...............................T.............  80
AF130422.1 ..............................G...............................T.............  80
CP024619.1 ..............................................................................  80
CP018657.1 ..............................................................................  80
CP022658.1 ..............................................................................  80

Majority   ATAGCTGTTCTCCGCACTGCCGCCTGGGCTCCGATCCTGACTATTCGCGCCGGTAAAGGTGTAATTCAATAGCAACGCAT
                    90        100       110       120       130       140       150       160
LT795114.1 ..............................................................................  160
Egy strain ..............................................................................  160
CP022497.1 ..............................................................................  160
CP022491.1 ..............................................................................  160
LT571437.1 ..............................................................................  160
CP014977.1 ..............................................................................  160
CP014975.1 ..............................................................................  160
CP011233.1 ..............................................................................  160
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CP014356.1 ..............................................................................  160
LN999997.1 ..............................................................................  160
CP019383.1 ..............................................................................  160
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CP018648.1 ..............................................................................  160
CP016754.1 ..............................................................................  160
CP022003.1 ..............................................................................  160
CP018659.1 ..............................................................................  160
CP018655.1 ..............................................................................  160
CP018635.1 ..............................................................................  160
CP018633.1 ..............................................................................  160
CP017232.1 ..............................................................................  160
AF129435.1 .........T....................................................................  160
AF130422.1 .........T....................................................................  160
CP024619.1 ..............................................................................  160
CP018657.1 ..............................................................................  160
CP022658.1 ..............................................................................  160
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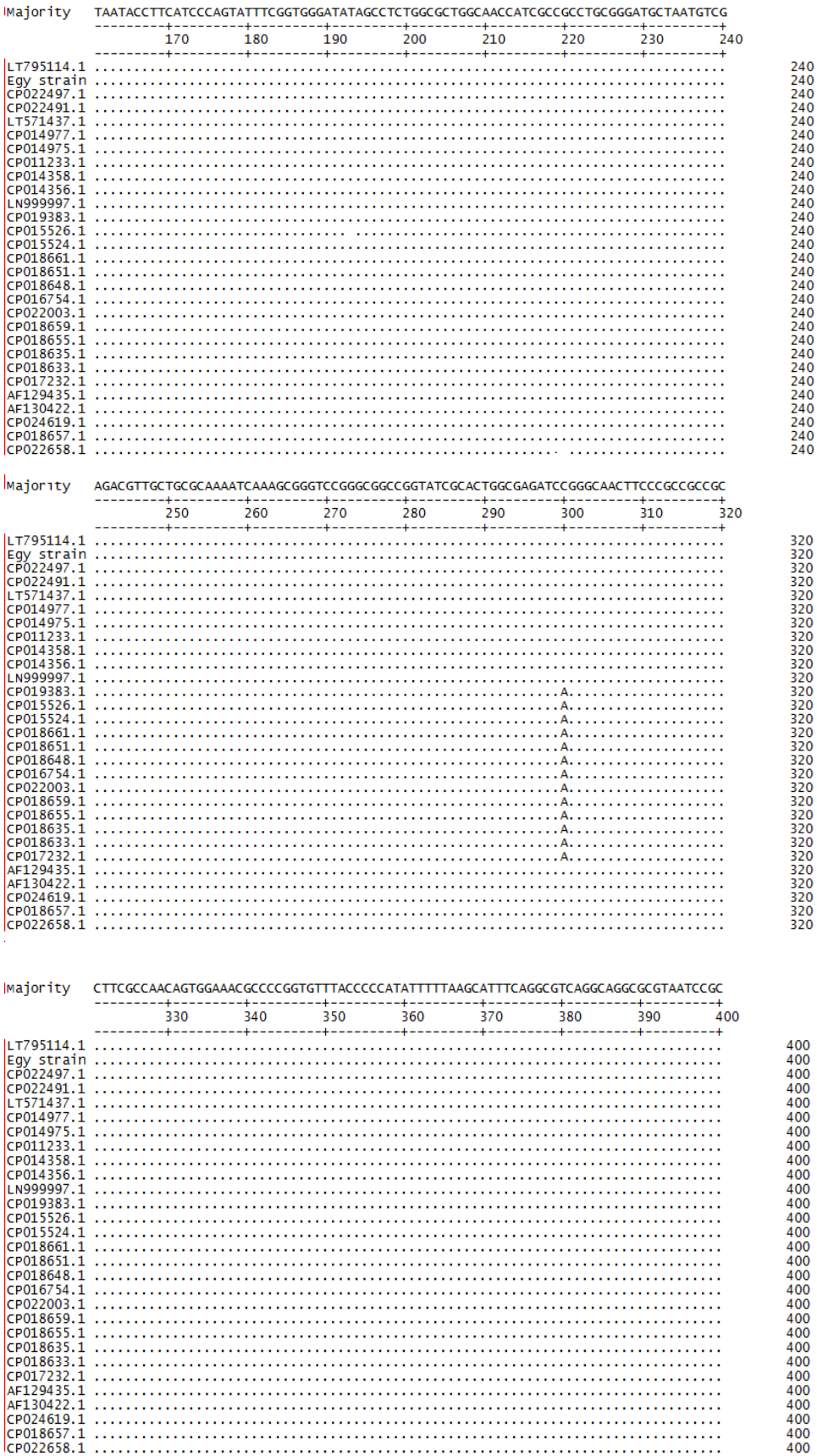

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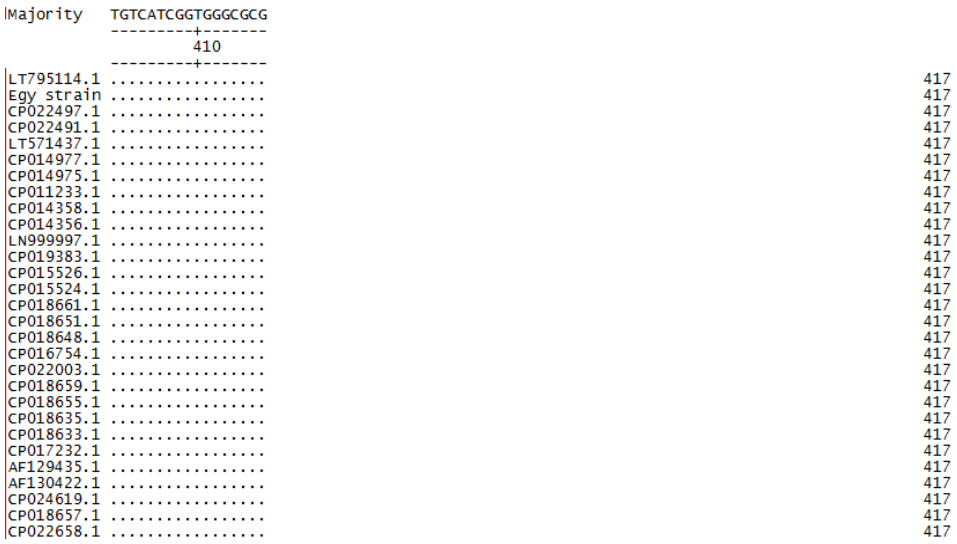

Figure 8. Nucleotides alignment of *bcfC* virulence gene sequence of Egyptian strain of *Salmonella* Typhimurium and different *Salmonella* Typhimurium strains retrieved from GenBank using CLUSTALW multiple sequence alignment program version 1.83 of MegAlign module of Lasergene DNASTAR.

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, AbeerMwafy and Hala Ahmed Al-Sawy designed and performed study design. All authors shared in writing, and approved the final version of manuscript.

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Competing interests

The authors declared that no competing interests exist.

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