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Research paper

Genomic features of colistin resistant *Escherichia coli* ST69 strain harboring *mcr-1* on IncHI2 plasmid from raw milk cheese in Egypt

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ABSTRACT

There is emerging evidence that food of animal origin may be responsible for the spread of multidrug resistant extraintestinal pathogenic *Escherichia coli* in the community. Here, we describe the emergence of colistin resistance gene, *mcr-1*, in a strain belonging to the dominant uropathogenic *E. coli* ST69 lineage. *E. coli* strain CFSAN061770 was isolated during monitoring of the popular Egyptian raw milk cheese, karish cheese, for the presence of colistin resistance. The complete genome of *E. coli* strain CFSAN061770 comprises a chromosome of 5,292,297 bp with a G + C content of 50.6%. Further, three plasmids named pEGY1-MCR-1, pEGY2 and pEGY3 of 228,947 bp, 103,234 bp and 87,012 bp were detected, respectively. Plasmid pEGY1-MCR-1 belongs to the IncHI2 incompatibility group and carries the colistin resistance *mcr-1* gene flanked by two IS*Apl1* elements and forms a composite transposon. It mediates resistance to aminoglycosides (*aadA1* and *aadA2*), phenicol (*cmlA1* and *floR*), sulfonamides (*sul3*), and tetracycline (*tet*(*A*)), and these loci were found clustered in a multidrug resistant region. Plasmid pEGY3 carries a complex multiple resistance locus (CMR) (*aph(3′)-Ia, strA, strB, sul2*, and bla_{TEM-1}) encoding resistance to different classes of antibiotics. Interestingly, the closest plasmids to plasmid pEGY1-MCR-1 detected from the NCBI Blast search belonged to the incompatibility group IncHI2 and were from the Kingdom of Saudi Arabia and Qatar which suggests a dissemination of pEGY1-MCR-1-like plasmids in the Middle East. Most striking, and of great public health concern is that strain CFSAN061770 carries five virulence genes (*iss, fimH, iutA, kpsMIII* and *kpsTIII*) which were identified in clinical extraintestinal pathogenic *E. coli*. Besides that, it carries the *astA* gene, which codes for the enteroaggregative *E. coli* heat-stable toxin 1 (EAST1).

1. Introduction

The increasing role of colistin in humans as a last-resort antibiotic against lethal infections caused by carbapenem-resistant Gram-negative pathogens, has encouraged more careful monitoring of resistance to this polypeptide (Kempf et al., 2016). Since the first discovery of the mechanism of colistin resistance mediated by *mcr-1* gene in late 2015, *mcr-1* has already spread to over 40 countries/regions covering five of seven continents (Sun et al., 2018). It has been elucidated that the transferability of colistin resistance seems to be mediated by different MCR-like enzymes, namely *mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, and mcr-8* (Partridge et al., 2018; Wang et al., 2019). This suggested that *mcr-1* has been evolved under some unknown selective pressure in the environment, animals, and humans (Sun et al., 2018).

Several studies have confirmed the transmission of *mcr*-gene *via* epidemic plasmids among Enterobacteriaceae isolated from animals, animal products human fecal samples, and the environment (Matamoros et al., 2017). Overall, the different replicon types are categorized into IncI2, IncHI2, IncP, IncX4, IncY, IncF, IncFI, IncFII, IncFIB, IncK2, including hybrid versions like IncX3-IncX4 and IncI2-IncFIB (Feng, 2018). Interestingly, the genomic comparison of different plasmid sequences originated from diverse host strains isolated from different geographic locations showed an immediate background common to all *mcr-1* sequences, implying a single common origin for all *mcr-1* elements sequenced to date (Wang et al., 2018). It is hypothesized that an initial single mobilization event of *mcr-1* by an IS*Apl1-mcr-1-orf*-IS*Apl1* transposon happened around 2006 followed by stabilization of *mcr-1* in a diverse range of plasmid backgrounds after losing the

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flanking IS*Apl1* elements and subsequently, spread through plasmid transfer (Wang et al., 2018).

One of the main mechanisms involved in the spread of antimicrobial resistance is the dissemination of specific clones that acquire antimicrobial resistance genes. *Escherichia coli* clonal group A (CgA) which belongs to sequence type (ST) 69 and exhibits O groups O11, O17, O73 and O77, is a good example of such clones (Manges et al., 2001). Since its first recognition in 1999 in Northern California, USA, CgA or ST69 strains have been reported globally and were isolated from urinary tract and blood stream infections (Riley, 2014). One major concern is the evolution of antimicrobial resistance in ST69 strains worldwide (Giacobbe et al., 2015; Wang et al., 2016). The CgA strains are progressively gaining new antimicrobial resistance genes, including extended-spectrum beta-lactamases (ESBLs) and carbapenemases, revealing alarming antibiotic resistance accumulation (Giacobbe et al., 2015; Wang et al., 2016). To date, the *mcr*-1 gene which confers resistance to colistin, the last-resort drug for some multidrug-resistant (MDR) infections, has been found in different *E. coli* strains belonging to diverse STs worldwide (Poirel et al., 2017).

Globally, there is a few genomic data about colistin resistant *E. coli* from ready to eat food. Additionally, there is a lack of studies about the virulome of *E. coli* ST69 from food of animal origin. In this study, we describe the genomic features of colistin resistance gene, *mcr-1*, in a strain belonging to the dominant uropathogenic *E. coli* ST69 lineage isolated from popular Egyptian raw milk cheese, karish cheese.

2. Materials and methods

2.1. Bacterial strains and identification

During the period of December 2016 to February 2017, we cultured *E. coli* strains from two hundred samples (100 g each) of karish cheese collected from Egypt in a study aiming to detect the incidence and molecular characteristics of colistin resistant *E. coli* in this popular type of raw milk cheese at the Department of Food Hygiene and Control, Faculty of Veterinary Medicine, University of Sadat City, Egypt. Cheese samples (25 g) were diluted with 225 ml of buffered peptone water (Oxoid, Basingstoke, England), homogenized in a stomacher, seeded onto eosin methylene blue agar (Oxoid, Basingstoke, England) plates supplemented with 2 mg/l colistin, and incubated for 24 h at 37 °C. Colonies grown on eosin methylene blue agar plates were identified by traditional biochemical tests. Strains confirmed as *E. coli* by the API 20E system (bioMe'rieux, Marcy l'Etoile, France), were shipped to the Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, Maryland, USA. On arrival at the FDA laboratory, *E. coli* strain CFSAN061770 was selected for sequencing.

2.2. DNA extraction and genome sequencing

The genomic DNA of strain CFSAN061770 was isolated from an overnight culture using the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) and sequenced on the Pacific Biosciences (PacBio) RS II sequencing platform as previously described (Yao et al., 2016). The sequences were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html) and deposited at DDBJ/EMBL/GenBank.

2.3. Genomic and comparative analyses

Sequences were analyzed *in silico* for multilocus sequence type (MLST), antimicrobial resistance and virulence genes profiles and plasmid replicon types using MLST 1.8, ResistanceFinder, VirulenceFinder, and Plasmid finder database of the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/) (Carattoli et al., 2014; Joensen et al., 2014; Larsen et al., 2012; Zankari et al., 2012). Furthermore, we investigated the presence of 30 ExPEC virulence genes contents of our strain and a representative collection of 93 *E. coli* ST69 strains (Supplemental Table S1) by using BLASTN against previously described reference genes (Johnson and Stell, 2000). Through Blast Score Ratio (BSR) analysis (Sahl et al., 2014), we compared the prevalence and distribution of virulence genes in *E. coli* ST69. Circular map of plasmid pEGY1-MCR-1 (accession number CP023143) compared to two other closest mcr-1 plasmids in NCBI (pSA186_MCR1, accession number CP022735) and pMS8345A, accession number CP025402), generated using CGView (Grant and Stothard, 2008).

2.4. Phylogenetic analysis

To understand the evolutionary context of this strain we constructed a phylogenetic tree using the genomes of 93 additional *E. coli* ST69 strains available in the NCBI database (Supplement Table S1). The phylogenetic relationship of the strains was assessed by a custom cgMLST analysis using Ridom SeqSphere + software v4.0 (http://www.ridom.com/seqsphere). The genome of *E. coli* O157:H7 strain Sakai (NC_002695) served as a reference (5204 genes). Another six genomes were used for comparison (Query) to determine the core and accessory genes in the Sakai genome. These six genomes were: four *E. coli* O157:H7 genomes (NC_011353.1 strain. EC4115, NC_002655.2 str. EDL933, NC_013008.1 strain TW14359, and NC_017906.1 strain Xuzhou21) and 2 *E. coli* O55:H7 genomes (NC_013941.1 O55:H7 strain CB9615, and NC_017656.1 O55:H7 strain RM12579). After this analysis, 3860 genes were defined as core genes (3,779,656 bases), 791 genes as accessory genes (536,551 bases), and 553 genes were discarded (present in > 1 copy in the genome, or were pseudogenes). A total of 4651 genes were available for analysis. The genomes of 94 *E. coli* were blasted against these genes and only 2409 were present in all the strains. A maximum likelihood tree using the appropriate genetic distances was built after the cgMLST analysis. The discriminatory index was calculated with the Ridom software using the Simpson's discriminatory index as previously described (Hunter and Gaston, 1988).

3. Results

3.1. Identification of mcr-1-Positive E. coli Isolates

In our study, four colistin resistant *E. coli* isolates were collected from 200 samples of ready to eat karish cheese. *E. coli* strain CFSAN061770 was selected for sequencing.

3.2. Genomic analyses

In silico genotyping revealed that *E. coli* strain CFSAN061770 belongs to ST69 and O17/O44:H18 serogroup. The complete genome of *E. coli* strain CFSAN061770 comprises a chromosome of 5,292,297 bp with a G + C content of 50.6%. Further, three plasmids named pEGY1-MCR-1, pEGY2 and pEGY3 of 228,947 bp, 103,234 bp and 87,012 bp were detected, respectively. Plasmid replicons IncHI2 and IncHI2A were detected in pEGY1-MCR-1, whereas IncQ1, IncFIB and IncFIA were detected in pEGY3. No replicon type could be detected in pEGY2 (unidentified Inc. group) (Shintani et al., 2015).

A large complement of plasmid encoded antimicrobial resistance genes was identified. Plasmid pEGY1-MCR-1 carries the colistin resistance *mcr-1* gene flanked by two IS*Apl1* elements and forms a composite transposon. The MDR region includes a class 1 integron, In641 (*estX-3, psp, aadA2, cmlA1, aadA1a*, and *qacH2*) that mediates resistance to aminoglycosides (*aadA1* and *aadA2*), phenicol (*cmlA1*), and quaternary ammonium compounds (*qacH2*). Antimicrobial resistance genes, *sul3* (sulfonamides), *floR* (phenicol) and *tet*(*A*) (tetracycline) were also detected in this region. Plasmid pEGY3 carries a complex multiple resistance locus (CMR) that carries genes encodes resistance to different antibiotics; *aph(3′)-Ia* (kanamycin and neomycin), *strA-strB* (streptomycin), *sul2* (sulphonamide), and $bla_{\text{TEM-1}}$ (ampicillin). In

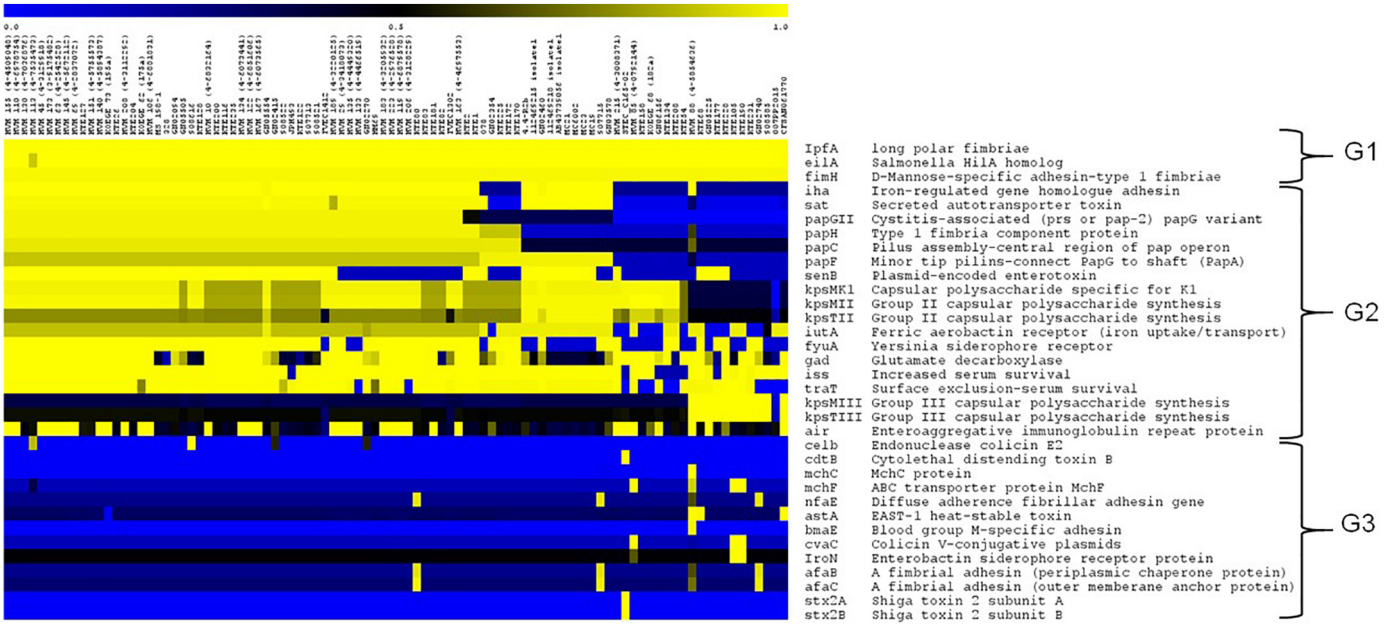

Fig. 1. Heat map indicating the BSR values for virulence genes in 94 *E. coli* ST69 strains. The presence or absence of the identified virulence gene inventory was determined using BLAST score ratio (BSR) analysis as previously described (Sahl et al., 2014). The protein-encoding genes that were considered present but divergent had BSR values of ≥0.4 and < 0.8, while those with BSR values of ≥0.8 were determined to be present with significant similarity. BSR values range from 0 (blue, absent) to 1 (yellow, identical). Gene inventories of groups, G1, G2 and G3 are discussed in the text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

addition, pEGY3 harboured a class 1 integron, In1449 with a gene cassette array *dfrA17* and *aadA5* that mediates resistance to trimethoprim and aminoglycosides, respectively. A transposon encoding *tet*(*A*), *tet*(*C*) and *tet*(*D*) (tetracycline) was also detected on pEGY3. The solely detected chromosomal-borne gene is an AmpC beta lactamase, bla_{EC}.

3.3. Distribution of virulence genes in E. coli ST69

As evident in the resulting heat map (Fig. 1) the virulence genes clustered into three distinct groups. Group 1, includes shared virulence genes that are present in all the strains; Group 2, miscellaneous virulence genes, includes genes that are present in different combinations in the majority of the strains; and group 3, rare virulence genes, includes genes that are present in only a few strains (less than four strains). All the strains lack the virulence genes *cnf1, focG, gafD, hylA, IbeA, PAI, papA, papE, PapGI, papGIII, rfc* and *sfaS* (data not shown). The identified virulence genes in our strain include *eilA, lpfA, gad, air, astA, iss, fimH, iutA, kpsMIII* and *kpsTIII* which belong to the three groups. All the virulence genes are carried on the chromosome except *iutA* which is carried on pEGY3.

3.4. Phylogenetic analysis

Phylogenetic analysis revealed that our strain clustered with *mcr-1* positive *E. coli* ST69 strain 007PP2015 from Poland (unpublished, accession number LWSE00000000) (Fig. 2).

4. Discussion

Colistin has been used in veterinary medicine for decades, for the prevention and treatment of Enterobacteriaceae infections and as an infeed additive to promote healthy development in food-producing animals. Unfortunately, in Egypt there is uncontrolled use of colistin in animals. Consequently, *mcr-1* gene was identified from strains isolated from human and animal (Elnahriry et al., 2016; Khalifa et al., 2016). Of note, little is known about the genomic features involved in the dissemination of *mcr-1* positive *E. coli* strains from food of animal origin in Africa and the Middle East. This study provides the first insights on the genomic features of an *E. coli* ST69 strain carrying the globally disseminated *mcr-1* gene from food of animal origin.

BLASTn search for pEGY1-MCR-1 identified two *E. coli* plasmids as closest phylogenetic relatives. Plasmids pSA186_MCR1 (accession CP022735) and pMS8345A (accession CP025402) originate from clinical cases isolated in the Kingdom of Saudi Arabia and Qatar (Fig. 3), respectively. *In silico* typing of these plasmids using PlasmidFinder revealed that they belongs to the same plasmid incompatibility group of our plasmid, IncHI2. Interestingly, a global phylogenetic analysis of *E. coli* and plasmids carrying the *mcr-1* gene revealed significant geographical clustering of different plasmid incompatibility groups carrying *mcr-1* gene with regional spread of IncHI2 plasmids in Europe and IncI2 in Asia (Matamoros et al., 2017). In shortage of information about complete nucleotide sequences of plasmids from Africa and the Middle East, the geographical clustering of different plasmid incompatibility groups carrying the *mcr-1* gene could not be addressed. In this study, as illustrated in Fig. 3, two IncHI2 plasmids carrying *mcr-1* detected from two different countries in the Middle East are nearly identical to our plasmid, pEGY1-MCR-1. Based on their shared geographic distribution with pEGY1-MCR-1, this plasmid type may play a particular role in disseminating the *mcr-1* gene in the Middle East.

The presence of the *mcr*-1 gene in a transposon is highly worrisome, since it enables accelerated dissemination of *mcr-1* by recombination of the whole transposon to either plasmids or chromosomes. We found that pEGY1-MCR-1 exhibits extensive similarity to the backbone of the *mcr-1* positive MDR plasmid pSA26-MCR-1 (accession number KU743384), which was recently isolated from a clinical sample in the Kingdom of Saudi Arabia (Sonnevend et al., 2016). Plasmid pEGY3 carries a complex multiple resistance locus (CMR) similar to plasmid pO26-CRL-125 (accession number KC340960), which was isolated from a clinical enterohemorrhagic *E. coli* strain in Australia (Venturini et al., 2013). It remains unclear to what extent plasmids carrying colistin resistance gene isolated from animals and food of animal origin helped to shape the landscape of the genomes of clinical colistin resistant strains.

As opposed to the analyzed strain CFSAN061770, which carries

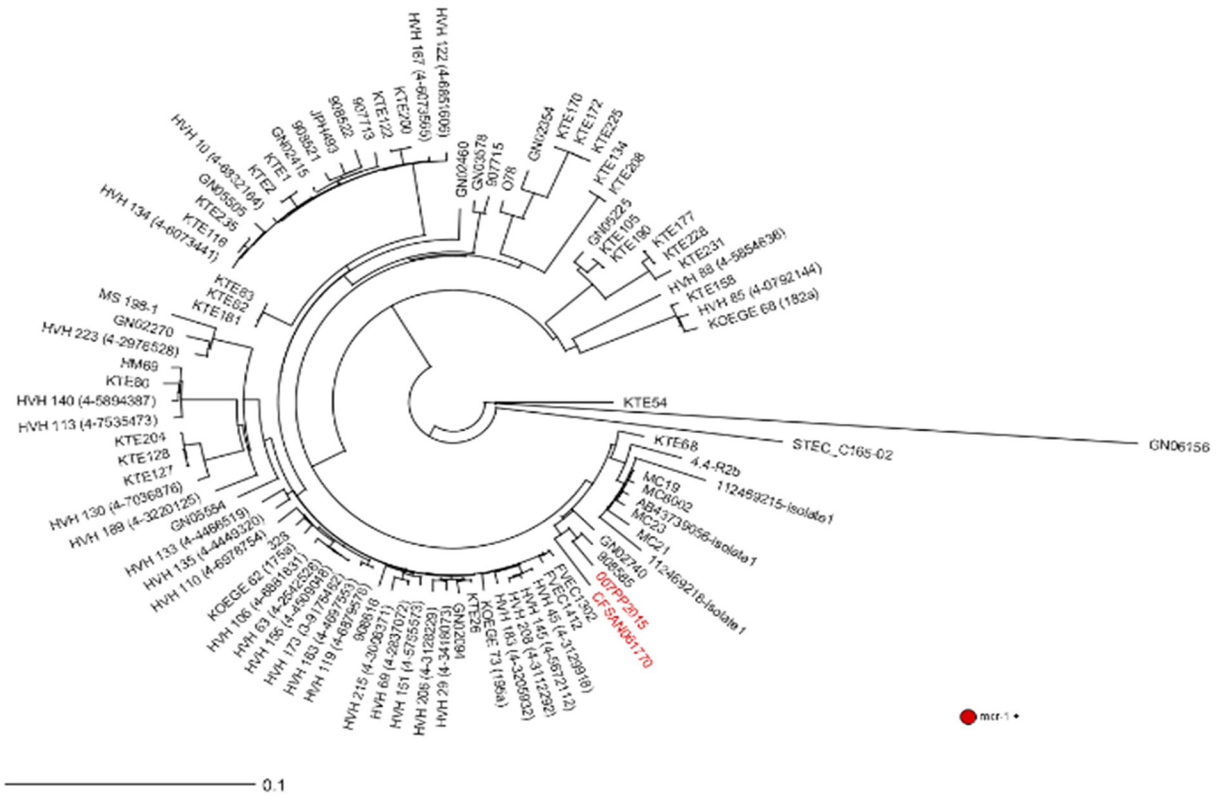

Fig. 2. Phylogenetic analysis of the *E. coli* ST69 CFSAN061770 strain carrying *mcr-1* gene compared to other ST69 strains from around the world (available at NCBI) based on cgMLST analysis.

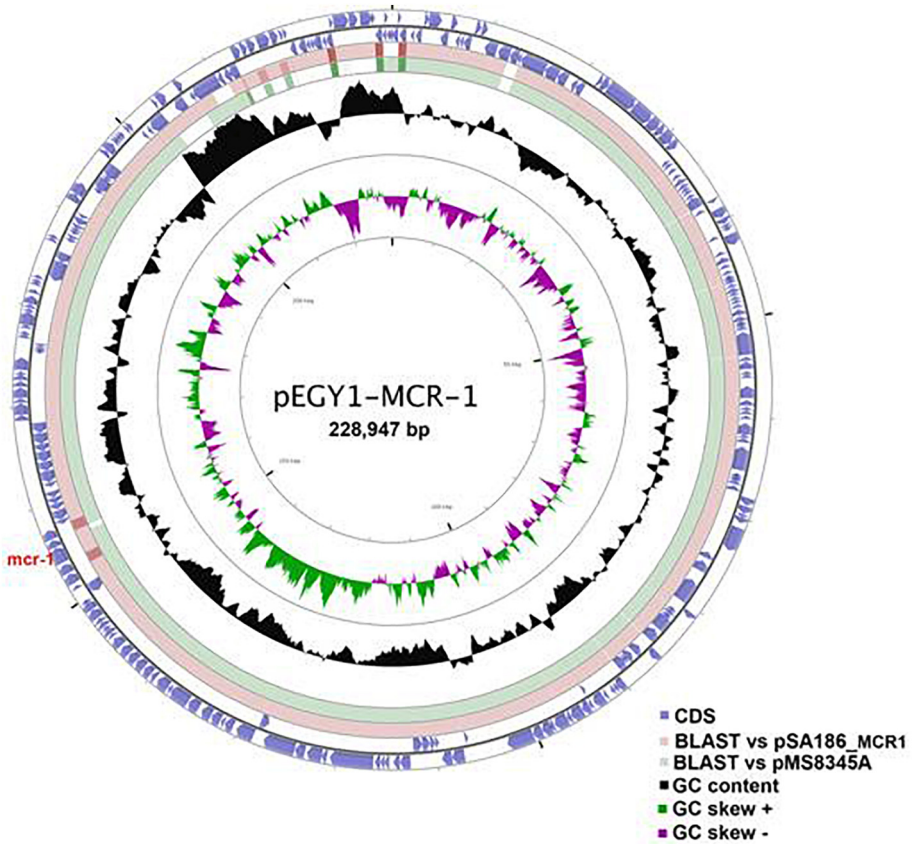

Fig. 3. Circular map of plasmid pEGY1-MCR-1 (accession number CP023143) compared to two other closest *mcr-1* plasmids in NCBI (pSA186_MCR1, accession number CP022735) and pMS8345A, accession number CP025402), generated using CGView (Grant and Stothard, 2008). Blue block arrows in the outer circle denote coding regions in the plasmid, indicating the ORF transcription direction. G + C content is shown in the middle circle and the deviation from average G + C content (46.1%) is displayed in the innermost circle. BLAST comparisons with the other two plasmids are shown in light red (pSA186_MCR1), and green (pMS8345A). The location of the *mcr-1* gene is shown in red fonts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resistance genes for traditionally used antibiotics including sulfonamides, phenicol, tetracycline, aminoglycosides and ampicillin, a number of studies in *E. coli* have reported on the presence of *mcr-1* gene together with ESBL, carbapenemase and quinolone resistance genes (Rhouma and Letellier, 2017). Strain CFSAN061770 is similar in its genotype to strains from the Mediterranean basin where such antibiotics are broadly used in veterinary medicine (Dandachi et al., 2018). Indeed, it appears that the emergence of *mcr-1* carriers in animals and food of animal origin in certain geographical areas is related to the co-selective pressure applied by the over usage of non-beta-lactams. Consequently, a comprehensive strategy to prevent the dissemination of *mcr-1* carriers from animal and food of animal origin to human should be based not only the reduction or banning of colistin use in livestock but also on the overall reduction of use of other antibiotics.

There is emerging evidence that food of animal origin may be responsible for the spread of extraintestinal pathogenic *E. coli* (ExPEC) in the community (Ramchandani et al., 2005). We identified the virulence gene contents of our strain and a strain panel from the data base. Most striking, and of great public health concern is that our strain carries five virulence genes (*iss, fimH, iutA, kpsMIII* and *kpsTIII*) which were identified in clinical ExPEC. In agreement with a previous study (Karami et al., 2017), carriage of *iutA* gene was associated with incidence of many resistance genes in our strain. Our finding emphasis the role of *iutA* gene as a strong predictors for antimicrobial resistance. Surprisingly, out of 93 *E. coli* ST69 strains investigated for 30 virulence genes (Fig. 1), our strain along with only 2 other strains (KTE80, HVH 88 (4–5,854,636)) carried the *astA* gene, which codes for the enteroaggregative *E. coli* heat-stable toxin 1 (EAST1) (Savarino et al., 1993). It is worth to mention that, the term "heteropathogenic *E. coli*" is now used to name pathogenic *E. coli* strains that carry virulence genes from different *E. coli* pathotypes (Ang et al., 2016). Importantly, in consistent with previous studies (Lara et al., 2017; Wallace-Gadsden et al., 2007), the identification of the *astA* gene in our strain give a new evidence that the ST69 complex has a propensity to acquire EAEC virulence genes and therefor it may have evolved as a progenitor lineage from which uropathogenic *E. coli* and EAEC strains belonging to ST69 emerged.

Recently, a core genome multilocus sequence typing approach (cgMLST) was proposed as a standard method for differentiation of strains based on whole genome sequencing. As it depends on core genes, it provides an accurate, efficient, and reproducible method for epidemiological investigation. In this study, the primary investigation of the phylogenetic tree (Fig. 2), revealed that the Egyptian strain clustered with *E. coli* ST69 strain 007PP2015 which was isolated from a domestic turkey, *Meleagris gallopavo* in Poland. It showed resistance and virulence profiles similar to the ones detected in our strain but in different genetic context (data not shown). Interestingly, the driven force for the evolution of colistin-resistance in certain clones is still unclear and it is hypothesized that it may be elaborated as a part of adaptive evolution of bacteria with certain biological characteristics to some environmental and ecological factors. However, the close distance between our strain and the solely detected *mcr-1* positive *E. coli* ST69 strain isolated from turkey in Poland favors the hypothesis that certain clones of *E. coli* ST69 sharing a common genetic pool and circulating in animals in different geographical regions under different environmental and ecological factors have the propensity to acquire the *mcr-1* gene as a part of its resistance evolution.

In conclusion, this study sheds the light on the potential role of food of animal origin as an important reservoir of *mcr-1* containing bacteria, the important role played by IncHI2 in the dissemination of *mcr-1* and the occurrence of pEGY1-MCR-1-like plasmids in different countries in the Middle East. Strain CFSAN061770 carries multiple antimicrobial resistance genes located in plasmids with multiple replicon genes which may allow them to transfer and adapt to a broader host range. The fact that we identified a potentially virulent *mcr-1* positive food isolate belonging to a dominant lineage of urinary tract infection, *E. coli* ST69, from raw milk cheese in Egypt is worrisome and highlights the need for global surveillance addressing the presence of virulence and antimicrobial resistance gene in strains isolated from food of animal origin, especially those foods that are uncooked.

Data deposition

The complete nucleotide sequences of *E. coli* strain CFSAN061770 chromosome, pEGY1-MCR-1, pEGY2 and pEGY3 have been deposited into GenBank under the accession numbers of CP023142, CP023143, CP023144, and CP023145, respectively.

Acknowledgments

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.04.021.

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