Research Article

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Urinary tract infection attributed to *Escherichia coli* isolated from participants attending an unorganized gathering

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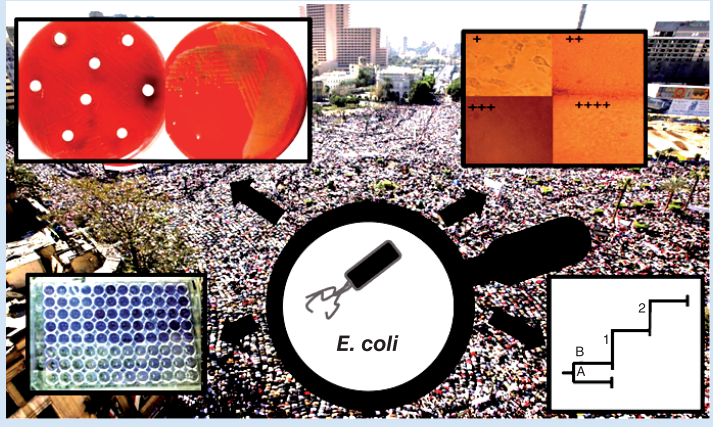
Aim: Participants in an unorganized gathering are potential hosts of diseases, bringing diseases from around the world to be introduced to a large at-risk population. Therefore, we investigated the gene repertoire in 29 *Escherichia coli* strains linked to urinary tract infection isolated from patients transferred to the hospital after attending an unorganized gathering in Cairo. **Materials & methods:** Virulence and resistance determinants, phenotypic antibiotic resistance, biofilm formation, their serotypes and phylogenetic relationships were analyzed. **Results:** The 29 tested serovars were phenotypically virulent, with the prevalence of group B2, and resistant to tetracycline, naldixic acid, ampicillin, trimethoprim, neomycin, oxytetracycline and erythromycin encoding the *iss* virulent gene. **Conclusion:** A One Health approach is a must to monitor and control *E. coli* urinary tract infections.



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Every year, millions of people travel internationally for either spontaneous or preplanned mass gatherings (MGs) that range from major sports events to fairs, festivals, concerts, religious and educational activities and political rallies [1,2]. Previous reports suggested that the MG can include as few as 1000 people or more than 25,000

Future Medicine

people [3]. This definition might not express an accurate estimation, as the main scenario evident on the ground is a consequence of sudden population displacement. Supermarkets, airports, seaports, malls, and trading centers where more than 100,000 people are coming in and out on a daily basis with minimal stress have been implicated to be ideal locations for the definition of MGs [1,4,5]. These locations are ideal for the study of MGs because there are a large number of attendees gathered at a specific site, for a specific occasion or reason, and usually a known duration, and there is potential to overextend local resources[1].These situations of MGs create an unmatched menace to the escalation of zoonotic diseases [4,5].

In an estimated 14 out of 21 documented events, long-distance spread has repeatedly originated at international MGs by participants traveling back to their home country [6]. Zoonotic infections are documented infectious disease threats to public health, and are associated with MGs globally [7]. A report by the European Centre for Disease Prevention and Control included studies on several disease outbreaks caused by bacterial and viral pathogens during MGs such as at football Euro and World Cups, Olympic and Paralympic Games and religious festivals, including an outbreak of *Escherichia coli* O157 at a rock festival in the south west of England [8].

In addition to the microbiological risks, antimicrobial resistance (AMR) is aggravated by the unpremeditated dispersion of resistant bacteria globally through traveling, and recently through MGs [9]. The European Centre for Disease Prevention and Control expressed their great concern [8] for the travelers attending the 2016 Olympics and Paralympics Summer Games in Rio de Janeiro, Brazil, regarding the risk of colonization of multi-drug resistance (MDR) Enterobacteriaceae. Yet no study was recorded with respect to this concern, giving the current study its novelty in being the first that gave this matter of the risk of the dispersion of MDR *E. coli* during an MG the importance of an investigation.

Therefore, we pursued the present study to investigate MDR *E.coli* using molecular and epidemiological characterization methods including: prevalence, phylogenetic relationships, virulence factors, and AMR genes. This study became the first investigation of its kind globally, as it took place in Egypt during the 2011 MG where hundreds of thousands of protestors had a continuous presence in Al Tahrir square for months, living their lives in tents or directly under the sky; eating, chatting, praying, selling, and so forth, but, most importantly, practicing prefiguration. During this period, El-Tahrir square assumed the form of a giant protest camp setting, carrying on living its own self-sustainable life, where street-stores and merchants were quite prominent. Protesters from all walks of life were scattered across the square: from street vendors selling sandwiches, pastries, and hot and cold drinks, to protesters, farmers, and hijab-wearing women, to young children, university students, and tourists. Even marriage ceremonies were performed. The square became a real city within a city with its own rules, codes, and infrastructure, including tents, clinics and all kinds of food and drink premises. It was without any medical and health professionals, veterinarians, food safety specialists or other experts to monitor and control diseases and infections, including *E. coli,* a common GI and urinary tract infection (UTI).

Materials & methods

Origin & characteristics of the bacterial strains studied & ethics statement

Kasr AlAini is a research and teaching hospital in Cairo, Egypt. This hospital is affiliated with the Faculty of Medicine, University of Cairo, with a capacity of more than 5200 beds and growing, in addition to 35 sections in different medical specializations. It is located 2.2 km south of El-Tahrir Square, the core of the MGs during 26 January 2011 to 31 March 2011 and where male patients with UTIs (i.e., >1 of the following symptoms: frequent urination; painful urination; hematuria; cloudy urine; or pain in pelvic area, flank, or low back and fever) were sent to the outpatient clinic for sample collection and isolation of *E. coli* in the Clinical Microbiology Laboratory (CML) of the hospital. A midstream urine capture at the hospital from patients with uncomplicated UTIs was immediately cultured after collection. The 29 clinical *E. coli* isolates in this study were re-identified by using the API 20E system (bioMérieux SA, Marcy l'Etoile, France). The *E. coli* included in the study were from cultures yielding $>10^5$ CFU/ml. The 29 clinical *E. coli* isolates had an instantaneous and overnight investigation for their phenotypic AMR. All isolates were propagated aerobically at 35°C in Luria-Bertani (LB) broth or on LB agar.

Somatic typing & expression of phenotypic virulence markers

The 29 available human *E. coli* isolates were tested for their pathogenicity [10]. Classical tests for *E. coli* pathogenicity included motility, hemolysin production, and Congo red uptake to differentiate between the virulent and avirulent *E. coli* isolates, assessment of chick embryo mortality (for infection, ~2 × 107 CFU/0.1 ml *E. coli*) and detection of cytotoxic activity on Vero cell monolayers and MDCK (Madin-Derby Canine Kidney) cells (for infection,

Table 1. List of PCR targets and oligonucleotide primers.

Gene targeted	Amplicon size (bp)	Primer sequences (5′–3′)	Function	Ref.
Primers for phylogenetic analysis				
chuA	279	F- GACGAACCAACGGTCAGGAT	Heme transport	[13]
		R- TGCCGCCAGTACCAAAGACA		
YjaA	211	F- TGAAGTGTCAGGAGACGCTG	Uncharacterized function	
		R- ATGGAGAATGCGTTCCTCAAC		
TspE4.C2	152	F- GAGTAATGTCGGGGCATTCA	Anonymous DNA fragment	
		R- CGCGCCAACAAAGTATTACG		
		R- CTA TTG TGA GCA ATA TAC CC		
Primers used for drug resistance genes				
tet(A)	888	F- GTGAAACCCAACATACCCC	Tetracycline resistance	[20]
		R- GAAGGCAAGCAGGATGTAG		
dhfrI	391	F- AAGAATGGAGTTATCGGGAATG	Trimethoprim resistance	
		R- GGGTAAAAACTGGCCTAAAATTG		
dhfrV	432	F- CTGCAAAAGCGAAAAACGG		
		R- AGCAATAGTTAATGTTTGAGCTAAAG		
dhfrXIII	294	F- CAGGTGAGCAGAAGATTTTT		
		R- CCTCAAAGGTTTGATGTACC		
sulI	822	F- TTCGGCATTCTGAATCTCAC	Sulphonamide resistance	
		R- ATGATCTAACCCTCGGTCTC		
sulII	722	F- CGGCATCGTCAACATAACC		
		R- GTGTGCGGATGAAGTCAG		

~2 × 107 CFU/0.1 ml). *E. coli* J96 (for hemolysin production), EHEC strain EDL 933 (for chick embryo lethality and cytotoxic activity) were used as positive controls. *E. coli* K-12 and *E. coli* DH5α were also used as a negative control in the chick embryo lethality and cytotoxic activity tests.

Serotyping of the *E. coli* isolates were performed by the agglutination test using commercially available O-antisera (Denka Seiken Co. Ltd., Tokyo, Japan).

Antimicrobial susceptibility testing

The 29 purified isolates were subjected to a panel of 15 antibiotic discs for the antimicrobial susceptibility testing. The antibiotic discs used were: nalidixic acid, tetracycline, doxycycline, gentamicin, neomycin, norfloxacin, ciprofloxacin, chloramphenicol, colistin sulphate, ampicillin, oxytetracycline, streptomycin, erythromycin, trimethoprim and sulphamethoxazole trimethoprim. Resistance was determined according to the interpretative standards of CLSI [11] against an *E. coli* standard (ATCC No. 25922).

Biofilm formation assay

Biofilm formation by *E. coli* was assayed by the two classical methods as previously described [12]. For biofilm analysis, 1×10^7 CFU of *E. coli* in 100 µl of LB broth (1×10^8 CFU/ml) was inoculated into the wells of a 96-well flat-bottom polystyrene microtiter plate grown at 37°C for 48 h and the attached biofilms were stained with 0.1% crystal violet and the absorbance was measured at OD_{595} using a microplate reader. On the other hand, a visible film lining the wall and the bottom of the borosilicate tubes is consistent with biofilm formation and the degree of biofilm formed was evaluated as 1 = weak/none, 2 = moderate and 3 = high/strong. The reference strain of positive biofilm producer *E. coli* ATCC 35218 was used as control.

Phylogenetic classification

The phylogeny of the 29 *E. coli* isolates was determined as detailed in Table 1. *E. coli* ATCC 25922 was used as positive PCR control.

DNA preparation & identification of the genes associated with virulence & antibiotic resistance

DNA for template in PCR reactions were generated using cell pellets from 100 µl of an overnight culture containing the *E. coli* isolates in LB broth (Difco) with constant shaking at 37°C. The pellets were resuspended in molecular-grade nuclease-free water and incubated at 95°C for 20 min. The lysates were centrifuged at 2000 × *g* for 5 min and the supernatants were frozen for use in PCR.

The isolates were tested for six different antibacterial resistant genes [*tet(A), dhfrI, dhfrV, dhfrXIII, sulI* and *sulII]* using a multiplex PCR–based assay (Table 1) using the conditions specified for each primer.

The 50 µl PCR reactions were performed with 3 µl of bacterial lysate, 5 µl of 10 × primer mix, 25 µl of GoTaq Colorless Master Mix (Promega Corporation, WI, USA), and the remain volume with molecular-grade nuclease-free water. The isolates were additionally examined for the presence of eight virulence genes (*uid*A, *pap*C, $iroN_{E.\ coli}$, *kps*MT II, *omp*A, *ibeA, eae*A and *iss*) by PCR amplification. The primer sets and PCR conditions were used as previously described and indicated in Table 2. The 50 µl 'hot start' method PCR reactions consisted of 10 µl of template DNA, 5 µM of primers, 25 mM MgCl, 10 mM dNTPs, 23 ng BSA, 1.25 U Taq DNA polymerase (Sigma, MO, USA), and the remaining volume with molecular-grade nuclease-free water. Amplified products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. Positive control strains of *E. coli* containing genes used in this study included: *E. coli* ATCC 8739, DSM 4618, DSM 8695, ATCC 10798, ATCC 67973, ATCC 77379, *E. coli* 2363, ATCC 69119, ATCC 87386 and ATCC 86980. A negative reagent control consisted of the PCR buffer and nuclease-free water were used.

Sequencing reaction & genetic analysis

Purification of the PCR products was carried out using QIAquick Gel Extraction kit (Qiagen, GmbH, Hilden, Germany) using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, CA, USA, cat-number 4336817) according to the instruction of the manufacturer as follows. The 20 µl consists of: 2 µl Big dye terminator v.3.1, 1 µl primer, 1–10 µl template according to quality of band and concentration of DNA, complete to total volume of 20 µl with PCR grade water. Performing gene sequencing was carried out using an Applied Biosystems 3130 genetic analyzer (Hitachi, Japan). For sequence analysis the Bioedit software was used to analyze the sequence of M gene of the isolates in this study. For Phylogenetic analysis the DNAstar MegAlign software was used for the alignment for the M sequence and phylogenetic analysis for these isolates using Clustal V method. Sequence distance was calculated to display the divergence and identity percent values of each sequence pair. The software MegAlign calculates divergence in relation to the phylogeny reconstructed between sequence pairs and percent identity compares sequence pairs directly excluding phylogenetic relationships. Calculated Residue Substitutions by MegAlign software predicted to have occurred to give rise to the sequence differences in the current alignment. The table may be useful in identifying unusual patterns of substitutions.

Results

Phenotypic virulence factors

Results included the identification of *E. coli* phylogenetic groups, serovar distribution and associated virulence genes combinations isolated during the MG in El-Tahrir Square between 2011 and 2013. Congo red assay was used as a phenotypic marker for the invasive and noninvasive *E. coli*. In the present study, all of the 29 tested serovars for the CR-binding affinities were 100% positive, although binding activity varied according to serotypes. The isolates were also able to produce cytopathic effects on the Vero and MDCK cells and had 100% motility, embryo lethality as well as hemolytic activity.

Distribution of phenotypic resistance to individual antimicrobial agents

The results of testing the AMR of the 29 *E. coli* isolates recovered from human samples, indicated that 29 out of 29 isolates (100%) were resistant to tetracycline, naldixic acid, ampicillin, trimethoprim, neomycin, oxytetracycline and erythromycin. On the other hand, only five out of 29 (17.2%) were resistant to colistin (Table 3). In other words, 24 isolates were susceptible to colistin.

Prevalence of phylogenetic groups

Our phylogenetic analysis identified different pathotypes. Three phylogenetic groups were recovered (Table 4). The distribution of the 29 *E. coli* strains among the three phylogenetic groups was B2 (n = 13/29, 44.8%), followed by

Table 2. List of PCR targets with primers sequences for *E.coli* virulence gene amplification.

Target gene	Function	Pathotype	bp		Primer sequence	Cycling conditions	Ref.
uidA gene of *E. coli* (Encodes GUS (β-D-glucuronidase) protein)	Encodes for b-D-glucuronidase	EHEC	468	F	5′ ATC ACC GTG GTG ACG CATGTC GC 3	95°C for 5 min Amplification (35 cycles of) 95°C for 30 s, 50°C for 1 min 72°C for 1 min final extension 72°C for 5 min	[14]
				R	5′ CAC CAC GAT GCC ATG TTC ATCTGC 3′		
				R	5′ CTA TTG TGA GCA ATA TAC CC 3′		
papC Chaperon usher protein pyelonephritis-associated pili	Pilus associated with pyelonephritis	UPEC	501	F	5′ TGATATCACGCAGTCAGTAGC 3′	94°C for 3 min Amplification (30 cycles of) 94°C for 1 min 58°C for 30 s 68°C for 3 min final extension 72°C for 10 min	[15]
				R	5′ CCGGCCATATTCACATAAC 3′		
$iroN_{E.coli}$ a Catecholate siderophore (salmochelin) receptor	Catecholatesiderophore (salmochelin) receptor	UPEC	847	F	5′ ATCCTCTGGTCGCTAACTG 3′	–	[16]
				R	5′ CTGCACTGGAAGAACTGTTCT 3′		
*kps*MT II Group II capsule antigens	Group II capsule antigens	MNEC	280	F	5′ CAGGTAGCGTCGAACTGTA 3′	94°C for 3 min Amplification (30 cycles of) 94°C for 1 min 54°C for 30 s 68°C for 3 min final extension 72°C for 10 min	[17]
				R	5′ CATCCAGACGATAAGCATGAGCA 3′		
ompA Outer membrane protein	Outer membrane protein	MNEC, EHEC	919	F	5′ AGCTATCGCGATTGCAGTG 3′	94°C for 3 min Amplification (30 cycles of) 94°C for 1 min 58°C for 30 s 68°C for 3 min final extension 72°C for 10 min	[16]
				R	5′ GGTGTTGCCAGTAACCGG 3′		
ibeA Invasion of brain endothelium	Invasion of brain endothelium	MNEC	342	F	5′ TGGAACCCGCTCGTAATATAC 3′	–	[16]
				R	5′ CTGCCTGTTCAAGCATTGCA 3′		
eaeA En-teropathogenic attachment and effacement	Enteropathogenic attachment and effacement	EPEC/EHEC	384	F	5′ GAC CCG GCA CAA GCA TAA GC 3′	94°C for 5 min Amplification (35 cycles of) 94°C for 1 min 54°C for 30 s, 72°C for 2 min, final extension 72°C for 10 min	[18]
				R	5′ CCA CCT GCA GCA ACA AGA GG 3′		
iss Increased serum survival gene	Increased serum survival gene	ExPEC	266	F	5′ ATG TTA TTT TCT GCC GCT CTG 3′	–	[19]
				R	5′ CTA TTG TGA GCA ATA TAC CC 3′		

A (n = 10/29, 34.5%) and D (n = 5/29, 17.2%). The phylogenetic group B1, classified as physiological microflora in the GI tract, was not detected.

Prevalence & combinations of the virulence genes among phylogenetic groups

The 29 *E. coli* isolates screened by PCR analysis indicated that there were three patterns for the distribution of the virulence genes encoding the *E. coli* isolates. A double combination of virulence genes were detected in the form of

Table 3. Distribution of sero- and phylogenetic groups, biofilm formation, virulence traits and resistance genes among human isolates.

Serotypes	Phylogenetic groups	Biofilm biding activity	Virulence genes	Resistance genes	Antibiotic resistance profile	
					Antibiotics profile	Total number of antibiotics
O44: K74	B2	Low glass- low plastic	*ompA- iss*	*tet (A)- dhfrI- dhfrXIII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- G- CL	14/15
O44: K74	A	Low glass- low plastic	*iss*	ND	NA- T- TR- E- N- A- O- SXT- NX	9/15
O44: K74	B2	Low glass- low plastic	*iss*	*sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C- G- CL	15/15
O44: K74	A	Low glass- low plastic	*iss*	*sulI*	NA- T- TR- E- N- A- O- CF- DO- C	10/15
O44: K74	A	High glass- High plastic	*iss*	*dhfrI- sulI*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C- G- CL	15/15
O44: K74	B2	Low glass- low plastic	*papC- iss*	*sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- C- G	13/15
O44: K74	B2	High glass- low plastic	*iss*	*tet (A)- dhfrI- dhfrXIII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- G	13/15
O55:K7	A	Low glass- low plastic	*ompA- iss*	*dhfrI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- S	10/15
O55:K7	B2	Low glass- low plastic	*ompA- iss*	*dhfrI- dhfrXIII- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C	13/15
O55:K7	D	Low glass- low plastic	*iss*	*sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S	11/15
O55:K7	D	Low glass- low plastic	*papC- iss*	*dhfrXIII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C	13/15
O111:K58	B2	Low glass- low plastic	*papC- iss*	*tet (A)- dhfrI- dhfrXIII- sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C- G	14/15
O111: K58	D	Low glass- low plastic	*iss*	*dhfrI*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C	13/15
O114:K90	A	Low glass- low plastic	*ompA- iss*	*dhfrI- dhfrXIII- sulII*	NA- T- TR- E- N- A- O- SXT- NX- S- DO- C- G	13/15
O114:K90	B2	Low glass- low plastic	*papC- iss*	*dhfrI- dhfrXIII*	NA- T- TR- E- N- A- O- SXT- NX- S- DO- C- G- CL	14/15
O114:K90	B2	Low glass- low plastic	*iss*	*dhfrI- dhfrXIII- sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- DO- C- G	13/15
O114:K90	B2	Low glass- low plastic	*papC- iss*	*tet (A)- dhfrI- dhfrXIII- sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C- G	14/15
O114:K90	B2	Low glass- low plastic	*iss*	*dhfrI- dhfrXIII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- G	13/15
O125:K70	A	High glass- low plastic	*ompA- iss*	*tet (A)- dhfrI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S	11/15
O125:K70	B2	Low glass- low plastic	*iss*	*dhfrI- dhfrXIII- sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C- G	14/15
O125:K70	B2	Low glass- low plastic	*iss*	*dhfrI- sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C- G	14/15
O125: K70	A	Low glass- low plastic	*papC- iss*	ND	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO	12/15
O125:K70	A	Low glass- low plastic	*papC- iss*	ND	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C- G	14/15
O125:K70	A	Low glass- low plastic	*iss*	*tet (A)*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- G	13/15
O125:K70	B2	Low glass- low plastic	*iss*	*tet (A)- dhfrI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- C- G- CL	13/15
O125:K70	D	Low glass- low plastic	*iss*	*dhfrI- sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- C	11/15
Untypable	A	Low glass- low plastic	*iss*	ND	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO- C	13/15
Untypable	B2	High glass- High plastic	*iss*	*sulI- sulII*	NA- T- TR- E- N- A- O- SXT- NX- CF- S- DO	12/15
Untypable	D	High glass- low plastic	*papC- iss*	*dhfrXIII*	NA- T- TR- E- N- A- O- SXT- NX- CF	10/15

A: Ampicillin; C: Chloramphenicol; CF: Ciprofloxacin; CL: Colistin; DO: Doxycycline; E: Erythromycin; G: Gentamicin; N: Neomycin; NA: Naldixic acid; ND: Not detected; NX: Norfloxacin; O: Oxytetracycline; S: Streptomycin; SXT: Sulpha-methoxazole-trimethoprim; T: Tetracycline; TR: Trimethoprim.

Table 4. Relationship between phylogeny and virulence of *E. coli* isolates.

Phylogenetic groups	Virulence genes							Total genes/29
	ompA	*papC*	*iroN*$_{E.coli}$	*kps*MT II	*iss*	*eae*	*ibeA*	
A 10/29 (34.5%)	3/10 (30%)	2/10 (20%)	ND	ND	10/10 (100%)	ND	ND	15/29 (51.7%)
B2 14/29 (48.3%)	2/14 (14.3%)	3/14 (21.4%)	ND	ND	14/14 (100%)	ND	ND	19/29 (65.5%)
D 5/29 (17.2%)	ND	2/5 (40%)	ND	ND	5/5 (100%)	ND	ND	7/29 (24.1%)
Total gene	5/29 (17.2%)	7/29 (24.1%)	ND	ND	29/29 (100%)	ND	ND	

ND: Not detected.

Table 5. Relationship between phylogenetic groups and drug resistance of *E. coli* isolates.

Antibiotic	Resistance genes	Resistant isolates (n = 29)	% Resistance	Phylogenetic grouping of *E. coli* isolates, and their resistance, according to phylogenetic groups		
				Group A (n = 10)	Group B2 (n = 14)	Group D (n = 5)
Tetracycline	*tet(A)*	7	24.1	2 (20%)	5 (35.7%)	ND
Sulphonamide	*sulI*	12	41.4	2 (20%)	8 (57.1%)	2 (40%)
	sulII	14	48.3	3 (30%)	10 (71.4%)	2 (40%)
Trimethoprim	*dhfrI*	17	58.6	4 (40%)	11 (78.6%)	2 (40%)
	dhfrV	ND	ND	ND	ND	ND
	dhfrXIII	12	41.4	1 (10%)	9 (64.3%)	2 (40%)

ND: Not detected.

iss + *omp*A genes in five out of 29 isolates (17.2%), while the two genes *pap*C + *iss* were detected in seven out of 29 (24.1%). The *iss* gene was consistant in all of the 29 *E. coli* isolates (100%). The distribution of the other virulence genes was as follows: *pap*C 7/29 (24.1%) and *omp*A 5/29 (17.2%). None of the *iro*$N_{E.coli}$, *kps*MT II, *ibe*A and *eae* genes were detected in any of the 29 *E. coli* isolates (Tables 3 & 4).

Table 4 represents the analysis of the virulence factors profiles among the phylogenetic groups of *E. coli* isolates. While the *iss* gene was detected with the highest prevalence in seropathotypes A, B2 and D (100%), the *eae, ibe*A, *iro*$N_{E.coli}$, and *kps*MT II genes were totally absent from the three phylogenetic groups that were identified in the 29 *E. coli* isolates. It was also observed that the *omp*A gene was not detected in isolates belonging to phylogenetic group D.

Prevalence & combinations of the antibiotic resistance genes among seropathotypes

As recorded in Tables 3 & 5, the trimethoprim *dhfrV* gene was not detected in any of the 29 *E. coli* isolates. From the 29 *E. coli* screened isolates, four out of 29 (13.8%) did not carry any of the six antibiotic resistance genes (*tet(A), dhfrI, dhfrV, dhfrXIII, sulI* and *sulII*).

Four different antibiotic resistance genes combinations (19/29, 65.5%) were seen (Table 5) in the form of:

Eight double (eight out of 29, 27.6%) gene combinations: (n = 2/29 (6.9%), *dhfrI- dhfrXIII*,; n = n = 2/29 (6.9%), *dhfrI- sulI*, Trimethoprim + Sulphonamide), (n = 4/29 (13.8%), *sulI- sulII* Sulphonamide), seven triple (seven out of 29, 24.1%) gene combinations: (n = 1 (3.5%), *tet (A)- dhfrI- dhfrXIII*; n = n = 2/29 (6.9%), *dhfrI- sulI- sulII*, Trimethoprim + Sulphonamide; n = n = 2/29 (6.9%), *dhfrI- dhfrXIII- sulII* Trimethoprim + Sulphonamide; n = n = 2/29 (6.9%), *tet (A)- dhfrI- sulII* Tetracycline + Trimethoprim + Sulphonamide), two quadruple (two out of 29, 6.9%) gene combinations: (n = n = 2/29 (6.9%), *dhfrI- dhfrXIII- sulI- sulII* Trimethoprim + Sulphonamide) and two pentadruple (two out of 29, 6.9%) gene combinations: (n = n = 2/29 (6.9%) *tet (A)- dhfrI- dhfrXIII- sulI- sulII* Tetracycline + Trimethoprim + Sulphonamide).

Our phylogenetic analysis identified different pathotypes. Each pathotype exhibited a unique combination of virulence factors that results in a distinctive pathogenic mechanism. The phylogenetic group D was observed in 5% of the human isolates. The relation between phylogenetic group and antibiotic resistance indicated that the isolates belonging to group D were more related with multiresistance than those belonging to other groups.

Associations between biofilm formation, seropathotypes, virulence & antibiotic resistance

Generally, of the 29 *E. coli* isolates, 21 isolates (72.4%) exhibited a weak adherence affinity to a glass and plastic surfaces (Table 3), while only two out of 29 isolates (6.9%) were high in their affinity for biofilm formation on glass and plastic surfaces. There was no evident special association between biofilm formation affinity, seropathotypes, virulence and antibiotic resistance.

Discussion

As far as infectious diseases go, the most substantial risk to attendants to MGs would be a gastrointestinal infection. The possibility of exposure to infectious diseases at MGs is substantial and needs to be thoroughly explored after a previous study on the epidemiological situation of *E. coli* in the same situation, location and periods [10]. *Escherichia coli* epidemiology has not been explored during MGs and this study viewed the role of gene repertoire in bacterial niche ecology, including the genomic bases of phenotypes that are directly linked with pathogenicity. As far as infectious diseases, the most substantial risk to attendants to MGs would be a gastrointestinal infection.

People attending MGs potentially bring diseases from their geographical origin and disseminate a disease, potentially rapidly, to other individuals who are at the MG being Nationals or foreigners [9]. Therefore, it should be highlighted as the increased risk of communicable disease transmission are often problematic aspects resulting from sudden population displacement [9]. In 1987, an outbreak of MDR shigellosis affected more than 50% of the attendees at the annual Rainbow Family Gathering in Nantahala National Forest, NC, USA [21]. The risks associated with MGs and its role in the propagation and dissemination of MDR *E. coli* could have a devastating effect on the attendees at the MGs. The significant population density confined within the limits of Tahrir Square alongside poor health and community planning services presented several environmental risks. This situation likely generated sanitation, hygiene and environmental problems and altered water accessibility and quality at the local level. Previous risk analysis of MGs and modes of transmission [22] included factors such as close person-to-person contact, age and sex of the participants, number of participants, location, the capacity limit of the location, as well as the source of food, particularly street vendors, as the primary reservoir of *E. coli.* In addition, several other health and exposure factors were examined, such as prospective insect and animal vectors, standard of the water and sanitation resources, the homeless situation, housing (i.e., tents), and environmental conditions such as the cold winter weather which strained the immune system's tolerance to combat infections. Other factors examined in the analysis of MGs and modes of transmission included systemic susceptibilities of the population resulting in contagious or infectious disease outbreaks, including indigenous infectious agents circulating in the general and participating population.

To our knowledge, this is the first study to evaluate the different *E. coli* pathotypes in MGs in Egypt. The *E. coli* serotypes isolated from our patients attending the unorganized MG revealed serovars O44:K74, O55:K7, 111:K58, O114:K90 and O125:K70 which differed from the previously isolated serovars O164:K, O126:K71 and O86:K61 that were collected and analyzed a year earlier from the same environment [10] reflecting the introduction of new serotypes in the environ of Kasr AlAini Hospital. In the present study, the isolates fell mostly into phylogenetic group B2, which belongs to the virulent pathotype of *E. coli* [20,23–27], which reflects the risk associated with traveling to attend unorganized or organized MGs. Diversity in the serotypes and phylogenetic analyses identified both in the present investigation, and in previous and coming investigations, reflect the differences between the geographically *diverse* communities and demographic variables [28,29].

Contributors to the spread of AMR are the physical spread of resistant bacteria during MGs, and the geographical spread of resistant bacteria [9] through MG attendees, who are potentially bringing diseases from their own particular geography and could introduce a disease into the MGs. MDR resistance among *E. coli* has been reported from different regions of the globe, and the rate of antibiotic resistance is higher in *E. coli* from recurrent UTIs [30] which has emerged as an important reservoir of resistance to cephalosporins, fluoroquinolones and SXT being the first-line antibiotics by the international clinical practice guidelines [31]. The percentages of *E. coli* strains resistant to sulfonamides and trimethoprim vary with the geographical location of the patients in USA, Korea, Turkey, Europe, Austria, Greece, Portugal, Sweden, UK, Portugal and Sweden [30,32]. Previous studies gave diverse accounts from different parts of the world on the most commonly encountered Trimethoprim-resistant genes in the context of medical practice (*s ull, sulll, dhfrI, dhfrV* and *dhfrXIII*). Their rates were found to be different from many findings in a number of UTIs studies from Syria, Lebanon, Denmark, The Netherlands, Korea, Australia, Spain, Portugal, France, Belgium, and Turkey which could be attributed to several factors [33]. In addition, tetracycline resistance is widespread among uropathogenic *E. coli* isolates from human infections in Iran [34] and the most common *tet* resistance mechanism in *E. coli* is the tetracycline efflux pumps, which exports the drug out of the cell one of which is the *tetA* found in 95% of the MDR *E. coli* isolates from UTIs in Nigeria [35]. Of the 29 *E. coli* isolates specified in our study, a total of $\geq$16 were MDR to penicillins, sulfonamides, cephalosporins, tetracyclines, and aminoglycosides which have attributed to the emergence and dissemination of AMR strains of *E. coli* [36,37], several other findings from this study are noteworthy in terms of their public health importance. One of which is the disturbing finding that, 96.5% (28/29) of the *E. coli* isolates were resistant to SXT. This drug combination is recommended for treating a range of human infections, including complicated UTI, acute uncomplicated cystitis, and pyelonephritis [38]. Virtually all SXT-resistant isolates from this study (27/29), however, were susceptible to ciprofloxacin which is an important antimicrobial for treating infections caused by sulpha-methoxazole-trimethoprim (SXT)-resistant *E. coli*. The emergence and dissemination of AMR in *E. coli* strains of serotype O111 isolated in the present investigation, could result in hindering the treatment of certain human UTI and gastrointestinal infections [36]. The variability in the frequencies of resistance genes between our results from the published articles on *E. coli* UTIs is not a country-to-country difference only, but rather, a country situation difference. AMR phenotype is a crude measure to adequately explain co-selection of antibiotic resistance genes since antimicrobial exposure does not appear to

coselect for all genes encoding a given phenotype. For example, *Rosengren et al.* showed a close association of *dhfrI* with *sul1* and *sul2* while *dhfrXIII* was not associated with any *sul* gene, meaning sulfonamide exposure selecting for resistance mediated by *sul1* or *sul2* would lead to greater abundance of *dhfrI*-mediated trimethoprim resistance but not *dhfrXIII*-mediated trimethoprim resitance [39]. Exploring how AMR genes are associated with each other and antimicrobial exposure may explain some observed increase in sensitivity to certain antibiotics when others are used [39].

Conclusion

The possibility of dissimation of novel resistance patterns into the environmental echo system during participating in an MG requires cooperation between medical and health professions, veterinarians, farmers, food safety specialists and other experts, to monitor and control *E. coli* UTIs [40–42]. Therefore, haemolytic and antibiotic resistant *E. coli* strains are emerging pathogens and their molecular typing is useful for surveillance purposes. To the best of our knowledge, this is the first pilot study to characterize hemolytic and antibiotic-resistant *E. coli* in unorganized MGs in Egypt and worldwide [8]. Further investigations are required, concerning the effects of antibiotic-resistant strains in MGs on the natural ecosystem, and on selection of resistance and its impact on human health [8].

Future perspective

The continuous incidents of OGMGs or even UNORGMGs nationally throughout the year without effective control has worsened bacterial contamination and their resistome in the echo system. Therefore, a molecular surveillance system must be adopted for other Enterobactereacea to give an insight on the current and everchanging situation in the resistance prevalence and dissemination between organisms, and to correlate the isolates from the different ecological niches.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The patients were provided with written and verbal information on the study goal, methods, advantages and consequences of the study. Each patient's file was treated independently and confidentially, and only the data analyst had access to secured data and patients' information.

Summary points

MGs could aggravate the problem of multidrug-resistant dissemination in Egypt

- The 29 *Escherichia coli* isolates were resistant to 46.7% of the tested antibiotics.

Virulence & invasive capacity of the *E. coli* isolates

- The isolates were also able to produce cytopathic effects on the Vero and MDCK cells, and had 100% motility, embryo lethality, hemolytic activity and 100% CR affinity.
- The *iss* gene was detected with the highest prevalence in seropathotypes A, B2 and D (100%).
- The *eae, ibe*A, *iro*$N_{E.coli}$ and *kps*MT II genes were totally absent from the three phylogenetic groups.
- None of the *iro*$N_{E.coli}$, *kps*MT II, *ibe*A and *eae* genes were detected in any of the 29 *E. coli* isolates.

Phenotypic antibiotic resistance profile

- The 29 isolates were 100% resistant to tetracycline, naldixic acid, ampicillin, trimethoprim, neomycin, oxytetracycline and erythromycin.

High prevalence of antibiotic-resistance genes

Four different antibiotic resistance genes combinations (19/29, 65.5%) were seen in the form of:

- Eight double (8/29, 27.6%) gene combinations: (n = 2/29 (6.9%), *dhfrI- dhfrXIII*,; n = n = 2/29 (6.9%), *dhfrI- sulI*, Trimethoprim + Sulphonamide), (n = 4/29 (13.8%), *sulI- sulII* Sulphonamide).
- Seven triple (7/29, 24.1%) gene combinations: (n = 1 (3.5%), *tet (A)- dhfrI- dhfrXIII*; n = n = 2/29 (6.9%), *dhfrI- sulI- sulII*, Trimethoprim + Sulphonamide; n = n = 2/29 (6.9%), *dhfrI- dhfrXIII- sulII* Trimethoprim + Sulphonamide; n = n = 2/29 (6.9%), *tet (A)- dhfrI- sulII* Tetracycline + Trimethoprim + Sulphonamide).
- Two quadruple (2/29, 6.9%) gene combinations: (n = n = 2/29 (6.9%), *dhfrI- dhfrXIII- sulI- sulII* Trimethoprim + Sulphonamide).
- Two pentadruple (2/29, 6.9%) gene combinations: (n = n = 2/29 (6.9%) *tet (A)- dhfrI- dhfrXIII- sulI- sulII* Tetracycline + Trimethoprim + Sulphonamide).

Absence of resistance traits

- The trimethoprim *dhfrV* gene was not detected in any of the 29 *E. coli* isolates.
- Four isolates remained negative for *tet (A)- dhfrI- dhfrXIII- sulI- sulII*.

Diversity in phylogenetic groups, sequence types & pulsotypes

- Three phylogenetic groups (A, B2 and D) were recovered with the prevalence of group B2 (44.8%).
- Our isolates showed typical identity (100%) with *E. coli* plasmid pVM01, *E.coli* APEC-O1-plasmid-ColBM, *E. coli* plasmid-p300iro, *E. coli iss*-gene, *E.coli* isolate-Beijing1-*iss*, *E. coli* isolate-Xinda-*iss*, *E. coli* isolate-Tianda-*iss*, *E. coli* isolate-Beijing3-*iss*, *E. coli* isolate-Guizhou1-*iss* and *E. coli* isolate-E18-*iss*..
- The percentage of identity reached 98.4% with *E.coli* DNA-*iss*-plasmid.
- While the percentage of identity reached its lowest degree 95.2% with *E. coli* CFT073.
- From the phylogenetic analysis of the *iss* gene, we found that there are two groups: A and B.
- The B group was divided into subgroups B1 and B2, and subgroup B2 included three arms a, b and c.
- The majority of isolates fell within phylogenetic group B2, which belongs to the virulent pathotypes of *E. coli.*

Conclusion

- This is the first pilot study to characterize virulent antibiotic resistant *E. coli* in urinary tract infection (UTI) attending MGs in Egypt and globally.
- The variability in the frequencies of resistance genes between our results from the published articles on *E. coli* UTIs does not reflect a country-to-country difference only, but rather, a country situation difference.
- The possibility of dissimation of novel resistance patterns in the environmental echo system during participation in an MG requires cooperation between medical and health professions, veterinarians, farmers, food safety specialists and other experts, to monitor and control *E. coli* UTI.

References

1. World Health Organization. *Public Health for Mass Gatherings: Key Considerations.* World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland (2015).
2. Government of Canada. activities and initiatives. Mass gatherings (large-scale events). https://travel.gc.ca/travelling/health-safety/mass-gatherings
3. Tam JS, Barbeschi M, Shapovalova N *et al.* Research agenda for mass gatherings: a call to action. *Lancet Infect. Dis.* 12, 231–239 (2012).
4. Centers for Disease Control and Prevention. *Mass Gathering Preparedness - A Global Health Security Victory for All at World Cup in Brazil.* Atlanta, GA, USA, 232–6348 (2014).
5. Elachola H, Gozzer E, Zhuo J *et al.* Mass gatherings: a one-stop opportunity to complement global disease surveillance. *J. Health Spec.* 4, 178–185 (2016).
6. Lee CV, Brunette GW, Gallagher NM. *Travel to Mass Gatherings.*Centers for Disease Control and Prevention Centers for Disease Control and Prevention. Atlanta, GA, USA, (2012).

7. World Health Organization. *Public Health For Mass Gatherings: Key Considerations.* World Health Organization, Geneva, Switzerland (2016).
8 . European Centre for Disease Prevention and Control. *Potential risks to public health related to communicable diseases at the Olympics and Paralympics Games in Rio de Janeiro, Brazil (2016).* Stockholm, Sweden (2016).
9. Al-Tawfiq JA, Memish ZA. Potential risk for drug resistance globalization at the Hajj. *Clin. Microbiol. Infect.* 21, 109–114 (2015).
10. Osman KM, Mustafa AM, Elhariri M *et al.* Identification of serotypes and virulence markers of *Escherichia coli* isolated from human stool and urine samples in Egypt. *Indian J. Med. Microbiol.* 30, 308–313 (2012).
11 . Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing 32(Suppl. 22) CLSI document M100-S22. Wayne, PA, USA (2012).
12. Nakao R, Ramstedt M, Wai SN *et al.* Enhanced Biofilm Formation by *Escherichia coli* LPS Mutants Defective in Hep Biosynthesis. *PLoS ONE* 7, e51241 (2012).
13. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic groups. *App. Environ. Microbiol.* 66, 4555–4558 (2000).
14. Heininger A, Binder M, Schmidt S *et al.* PCR and blood culture for detection of *Escherichia coli* bacteremia in rats. *J. Clin. Microbiol.* 37, 2479–2482 (1999).
15. Janßen T, Schwarz C, Preikschat P *et al.* Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. *Int. J. Med. Microbiol.* 291, 371–378 (2001).
16. Ewers C, Li G, Wilking H *et al.* Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? *Int. J. Med. Microbiol.* 297, 163–176 (2007).
17. Johnson JR, Stell AL. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *J. Infect. Dis.* 181, 261–272 (2000).
18. Wiles TJ, Kulesus RR, Mulvey MA. Origins and virulence mechanisms of uropathogenic *Escherichia coli*. *Exp. Mol. Pathol.* 85, 11–19 (2008).
19. Yaguchi K, Ogitani T, Osawa R *et al.* Virulence factors of avian pathogenic *Escherichia coli* strains isolated from chickens with colisepticemia in Japan. *Avian Dis.* 5, 656–662 (2007).
20. Maynard C, Bekal S, Sanschagrin F *et al.* Heterogeneity among virulence and antimicrobial resistance gene profiles of extraintestinal *Escherichia coli* isolates of animal and human origin. *J. Clin. Microbiol.* 42, 5444–5452 (2004).
21. Wharton M, Spiegel RA, Horan JM *et al.* A large outbreak of antibiotic-resistant shigellosis at a mass gathering. *J. Infect. Dis.* 162, 1324–1328 (1990).
22. World Health Organization. *Communicable disease alert and response for mass gatherings: key considerations (2008).* Publications of the World Health Organization can be obtained from WHO Press, World Health Organization, Geneva, Switzerland (2008).
23. Mokracka J, Koczura R, Jabłońska L *et al.* Phylogenetic groups, virulence genes and quinolone resistance of integron-bearing *Escherichia coli* strains isolated from a wastewater treatment plant. *Antonie van Leeuwenhoek* 99, 817–824 (2011).
24. Bashir S, Haque A, Sarwar Y *et al.* Virulence profile of different phylogenetic groups of locally isolated community acquired uropathogenic *E. coli* from Faisalabad region of Pakistan. *Ann. Clin. Microbiol. Antimicrob.* 11, 23 (2012).
25. Alizade H, Ghanbarpour R, Aflatoonian MR. Virulence genotyping of *Escherichia coli* isolates from diarrheic and urinary tract infections in relation to phylogeny in southeast of Iran. *Trop. Biomed.* 31, 174–182 (2014).
26. Lee S, Yu JK, Park K *et al.* Phylogenetic groups and virulence factors in pathogenic and commensal strains of *Escherichia coli* and their association with *bla*CTX-M. *Ann. Clin. Lab. Sci.* 40, 361–367 (2010).
27. Nandanwar N. Functional genotyping of extraintestinal pathogenic *E.coli* (ExPEC) belonging to the highly pathogenic Sequence type 95 reveals the zoonotic nature of human and avian strains. Inaugural-Dissertation To obtain the academic degree Doctor rerum naturalium (Dr. rer. nat.) Submitted to the Department of Biology, Chemistry and Pharmacy Of Freie University Berlin, (2014). https://d-nb.info/1049929845/34
28 . Manges AR, Johnson JR, Foxman B *et al.* Widespread distribution of urinary tract infections caused by a multidrug-resistant *Escherichia coli* Clonal Group. *N. Engl. J. Med.* 345, 1007–1013 (2001).
29. Wester AL, Melby KK, Wyller TB *et al. E. coli* bacteremia strains – high diversity and associations with age-related clinical phenomena. *Clin. Microbiol.* 3, 140 (2014).
30. Mellata M. Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. *Foodborne Pathog. Dis.* 10, 916–932 (2013).
31 . Abduzaimovic A, Aljicevic M, Rebic V *et al.* Antibiotic resistance in urinary isolates of *Escherichia coli*. *Materia Socio-Medica.* 28, 416–419 (2016).
32 . Wiedemann B, Heisig A, Heisig P. Uncomplicated urinary tract infections and antibiotic resistance – epidemiological and mechanistic aspects. *Antibiotics* 3, 341–352 (2014).

33. Al-Assil B, Mahfoud M, Hamzeh AR. First report on class 1 integrons and Trimethoprim-resistance genes from dfrA group in uropathogenic *E. coli* (UPEC) from the Aleppo area in Syria. *Mob. Genet. Elements* 3, e25204 (2013).
34. Gharajalar SN, Sofiani VH. Patterns of efflux pump genes among tetracycline resistance uropathogenic *Escherichia coli* isolates obtained from human urinary infections. *Jundishapur J. Microbiol.* e40884 (2017).
35. Igwe JC, Musa A, Olayinka BO *et al.* Tetracycline resistant genes in *E. coli* isolated from UTI and diarrhea patients in Zaria, Nigeria. *Clin. Microbiol.* 4, 225 (2015).
36. Schroeder CM, Meng J, Zhao S *et al.* Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. *Emerg. Infect. Dis.* 8, 1409–1414 (2002).
37. Doyle MP, Loneragan GH, Scott HM *et al.* Antimicrobial resistance: challenges and perspectives. *Comp. Rev. Food Sci. Food Saf.* 12, 234–248 (2013).
38. Karaca Y, Coplu N, Gozalan A *et al.* Co-trimoxazole and quinolone resistance in *Escherichia coli* isolated from urinary tract infections over the last 10 years. *Int. J. Antimicrob. Agents* 26, 75–77 (2005).
39. Rosengren LB, Waldner CL, Reid-Smith RJ. Associations between antimicrobial resistance phenotypes, antimicrobial resistance genes, and virulence genes of fecal *Escherichia coli* isolates from healthy grow-finish pigs. *Appl. Environ. Microbiol.* 75, 1373–1380 (2009).
40. Memish ZA. The Hajj: communicable disease alert and response for mass gatherings: key considerations. *Geneva, World Health Organization* (2008) www.who.int/csr/mass_gathering/en/index.html
41. Memish ZA. The Hajj: communicable and non-communicable health hazards and current guidance for pilgrims. *Euro Surveill.* 15(39), 19671 (2010)
42. AlNsour M, Fleischauer A. Public health considerations for mass gatherings in the Middle East and North Africa (MENA) region. *Eastern Mediterranean Health J.* 19, S42–S47 (2013).