Detection of gyrA mutation among clinical isolates of *Campylobacter jejuni* isolated in Egypt by MAMA PCR

Mayar M. Said1, Hanan El-Mohamady1, Fawkia M. El-Beih2, David M. Rockabrand1, Tharwat F. Ismail3, Marshall R. Monteville4, Salwa F. Ahmed1, John D. Klena4, Mohamed S. Salama2

1US Naval Medical Research Unit No.3, Cairo, Egypt
2Faculty of Science, Ain Shams University, Cairo, Egypt
3World Health Organization, Kabul, Afghanistan
4Present address: Naval Environmental and Preventative Medical Unit-2, Honolulu, HI, USA
5Present address: IEIP-CDC (China), Unit7300 Box050, DPO AP 96521-0060, USA

Abstract

Introduction: *Campylobacter* spp are the major cause of enteritis in humans and more than 90% of reported infections are caused by *Campylobacter jejuni*. Fluoroquinolones such as ciprofloxacin are the antibiotics of choice for treatment. An increase in the frequency of ciprofloxacin-resistant *Campylobacter* has been reported globally due to a single base mutation (C-257 to T) in codon 86 of the guanine resistance determining region (QRDR). *Campylobacter jejuni* gyrA gene altering the amino acid sequence from threonine at position 86 to isoleucine (Thr-86 to Ile).

Methodology: *Campylobacter* spp (n = 118) were selected from a collection of Egyptian isolates spanning 1998 to 2005. The presence of *C. jejuni* gyrA gene was confirmed in each isolate by a PCR assay amplifying 368bp portion of the gyrA gene. C to T alteration was detected by the mismatch amplification mutation assay MAMA PCR. The MIC of nalidixic acid (NA) and ciprofloxacin (CIP) was determined by E-test.

Results: *C. jejuni* gyrA gene was detected in 100 of the *Campylobacter* spp studied; the other 18 isolates were found to be *Campylobacter coli* by lpxA PCR. The mutation was detected in 89 *C. jejuni* resistant isolates with MIC values (NA; 8 – >256μg/ml) and (CIP; 4 - >32μg/ml). The other 11 sensitive *C. jejuni* isolates with MIC values (NA; 0.38 – 3μg/ml) and (CIP; 0.03 – 0.125μg/ml) were not amplified by the MAMA primers. There was 100% congruence with MAMA PCR, MIC results and gyrA gene sequence analysis.

Conclusions: In Egypt the main mechanism for resistance to fluoroquinolones is an alteration in the gyrA QRDR. MAMA PCR provides an economical and rapid means for screening fluoroquinolone resistance.

Key words: fluoroquinolone resistance, mismatch sequence detection, Africa


(Received 21 February 2010 – Accepted 07 May 2010)

Copyright © 2010 Said et al. This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

*Campylobacter jejuni*, and to a lesser extent *Campylobacter coli*, are recognized as major causes of bacterial gastroenteritis in developing and developed countries [1]. While most *Campylobacter* infections are self-limiting, antimicrobial agents may be prescribed for severe cases or in immunocompromised patients. The emergence of antimicrobial resistant *Campylobacter* may result in increased treatment times and treatment failure leading to increasing public health costs. Fluoroquinolones such as ciprofloxacin (CIP) are commonly used for the treatment of campylobacteriosis and have been recommended as a prophylactic treatment against travelers’ diarrhoea [2]. However, resistance of *Campylobacter* sp. to fluoroquinolones has been increasing worldwide [1,3,4].

*Campylobacter* resistance to fluoroquinolones has mainly involved alteration of the A subunit of the DNA gyrase enzyme, encoded by the gyrA gene, the target of this class of antibiotics [5]. A single nucleotide transition (C-257 to T) in the quinolone resistance determining region (QRDR) of the *C. jejuni* and *C. coli* gyrA alters the amino acid sequence of Gyra at position 86 from threonine to isoleucine (Thr-86 to Ile); this alteration is always associated with high MIC values for fluoroquinolones [6]. Various methods have been used to detect the gyrA C-257 to T transition, including denaturing gradient gel electrophoresis, [7] DNA sequencing of the target gene, [6] restriction fragment length polymorphism,
[8] non-radioisotopic single-strand confirmatory polymorphism and a fluorogenic PCR assay [9]. These methods are time-consuming, may require multiple steps, and often involve expensive equipment or reagents limiting their use in routine surveillance studies. A highly sensitive, simple, and rapid PCR technique known as mismatch amplification mutation assay MAMA PCR, [10] for the detection of this point mutation, was described by Zirnstein et al. [11]. MAMA PCR has been used to detect the C-257 to T nucleotide change without the need for DNA sequencing. The rationale for this method of mutation detection is to use a primer specific for the altered DNA sequence encoding the resistance that will exclusively generate a PCR product from resistant isolates. Empirically, an alteration at the 3’ end of the gyrA (MAMA) primer reduces the amplification efficiency to about 70%; however, a PCR product will still be produced. Introduction of a second mismatch immediately adjacent to the initial mismatch abolishes amplification of any DNA template [10,11].

In the present study, the MIC values for NA and CIP were determined for a set of clinical isolates of C. jejuni from Egypt and the MAMA PCR technique was used to detect the presence of the C-257 to T transition in the nucleotide sequence of the gyrA. Since this was the first application of the MAMA PCR technique in Egypt, sequencing of the amplified gyrA gene was conducted to confirm the sensitivity and specificity of MAMA PCR for gyrA as it applies to these isolates. We also examined the genetic background of the isolates using pulsed field gel electrophoresis to determine whether C. jejuni fluoroquinolone resistance was due to clonal expansion of numerous introductions into genetically diverse strains.

**Methodology**

**Bacterial culture and identification**

A total of 118 C. jejuni and C. coli isolates, recovered from rectal swabs collected from children under five years of age seeking medical treatment after providing a written informed consent from their guardians, were selected at random for this study. These samples were submitted to the bacteriology laboratory at NAMRU-3 as part of an active diarrhoeal surveillance project conducted at two different field locations in Egypt. For the active surveillance study, the study personnel visited each enrolled child at home twice a week and if a loose, liquid or bloody stool was reported by a parent, a fecal sample and/or rectal swab were collected from the child followed by a referral to a study physician for evaluation and treatment [12]. Isolates were initially recovered from 1998 to 2005, and no more than one isolate was taken from any single patient.

Rectal swabs were streaked onto Skirrow’s blood agar medium supplemented with vancomycin, polymyxin B, and trimethoprim. Inoculated plates were incubated at 42°C under microaerophilic conditions (5% O₂, 10% CO₂ and 85% N₂) for 48 hours. Suspected colonies were identified as Campylobacter on the basis of colony morphology, Gram stain, oxidase, and catalase reactions [12]. Hippurate and indoxyl acetate hydrolysis were used to identify Campylobacter to the species level [12]. Speciation was confirmed using the lpxA multiplex PCR [13]. This multiplex PCR assay was described by Klena et al. for the discrimination between the four thermotolerant Campylobacter species C. coli, C. jejuni, C. lari, and C. upsaliensis, which are the most commonly reported species in cases of Campylobacter gastroenteritis, using species-specific primers.

**Susceptibility testing**

The minimum inhibitory concentration (MIC) of Campylobacter isolates for CIP and NA were determined using E-Test strips following the manufacturer’s instructions (AB- Biodisk, Sölna, Sweden) and the guideline supplied by the Clinical and Laboratory Standards Institute (CLSI) [14]. The breakpoints for CIP (≥1mg/L) were determined as recommended by the CLSI [14] while NA breakpoints were determined as recommended by National Antimicrobial Resistance Monitoring System (NARMS), USA (≥32mg/L) [15]. C. jejuni ATCC33560 was used as a reference organism.

**DNA isolation, MAMA PCR, and sequencing**

Three to six colonies were picked from bacterial growth on Mueller- Hinton blood agar, and suspended into 0.1ml DNase/RNase free water (Sigma-Aldrich, Inc. St. Louis, MO, USA). Cell suspensions were boiled in a water bath for 10 minutes, chilled on ice for 30 minutes, and cell debris was removed by centrifugation at 13,000 x g. Supernatants containing DNA were carefully transferred to a sterile 1.5 ml Eppendorf tube and stored at -20°C until use [16].

PCR was performed as described by Zirnstein et al. [11]. A 368bp PCR product was amplified from
all C. jejuni isolates using the primer pair Campy MAMAgyrA1 (5'-TTT TTA GCA AAG ATT CTG AT-3') and GZgyrA4 (5'-CAG TAT AAC GCA TCG CAG CG-3'). MAMA PCR was performed using primers Campy MAMAgyrA1 paired with the mutation detection primer Campy MAMAgyrA5 (5'-CAA AGC ATC ATA AAC TGC AA-3'), amplifying a 265bp PCR product exclusively from the mutant gyrA alleles.

PCR amplicons generated with Campy MAMAgyrA1 and GZgyrA4 were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. Sequencing was performed using a Perkin Elmer Big Dye terminator Kit v3.1 (Applied Biosystems; Foster City, CA, USA) and an ABI PRISM 3100 automated sequencer. Data collected was analyzed using Applied Biosystems data collection software version 2.0, and sequencing analysis software 5.1.1. Nucleotide sequences were aligned using the Clustal X application in the BioEdit software package (Version 7.0.1) [17] and obtained sequences were compared to the sequence from a reference fluoroquinolone-sensitive strain C. jejuni (NCTC11168, GenBank accession number AL111168). MLST sequences were aligned using allelic sequences available from http://pubmlst.org/Campylobacter under the Campylobacter link. Allele numbers and a sequence type were assigned as described at this site.

Pulsed field gel electrophoresis

Macrorestriction profiling by pulsed field gel electrophoresis (PFGE) was performed as previously described [18]. After lysis, a 2 mm slice was cut from each washed plug and incubated overnight at 25°C with 200 µl of appropriate enzyme buffer containing 30U of Smal to digest the embedded DNA. Plug slices were loaded into preformed wells set in a 1% agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) prepared in 0.5X TBE. DNA from Salmonella enterica ss. enterica serotype Braenderup strain H9812 restricted with the enzyme XbaI (30U) served as the molecular mass standard. Smal mrp-PFGE patterns were stored and phylogenetic analysis of the DNA banding patterns was performed using BioNumerics (Applied Maths, Houston, TX, USA; software version 4.6).

Genetic analysis

A Kodak DC290 digital camera was used to capture 8-bit uncompressed TIFF images of each gel. Images were loaded into the BioNumerics software and DNA patterns were normalized by interpolation to the nearest reference lane. Optimization of 1.0% and a position tolerance of 1.25% were applied and a similarity matrix of the PFGE profiles from the studied isolates, based on the un-weighted pair group method with arithmetic averages (UPGMA), was calculated using Dice similarity coefficients.

Results

Fluoroquinolone-resistance of C. jejuni isolates determined by MIC and MAMA PCR results are congruent

Phenotypic analysis of 118 Campylobacter spp isolates, randomly selected from Campylobacter collected as a part of a study investigating causes of severe diarrhoea [12], indicated that 100 isolates were C. jejuni; the remaining 18 isolates were C. coli. Observed in vitro resistance to quinolones was very high; 89% (n = 89) of C. jejuni and 78% (n = 14) of C. coli isolates were resistant to NA and CIP. We analyzed changes in the gyrA gene of C. jejuni isolates as previously described [11]. A 368bp product of the C. jejuni gyrA gene was amplified in all C. jejuni isolates (Table). The C-257 to T mutation, as determined by the amplification of a 265bp PCR product, was detected in 89 isolates (89%) using MAMA PCR. C. jejuni isolates producing MAMA PCR positive results were also resistant to CIP (≥4mg/L) and had MIC values for NA of ≥8mg/L. In contrast, the remaining C. jejuni isolates were not amplified using the MAMA PCR and the MIC values with respect to NA and CIP ranged from 0.38 to 6.0mg/L and 0.032 to 0.125mg/L, respectively for the sensitive isolates. Although NARMS has set the breakpoint of NA at ≥32µg/ml, in the present study the codon 86 alteration was associated with MIC values for NA ≥8mg/L.

Since this was the first known application of the MAMA PCR method for detection of fluoroquinolone-resistance in C. jejuni isolates in Egypt, DNA sequencing of a 368bp gyrA amplicon was performed to confirm the presence of the ACA to ATA mutation at gyrA nucleotide position 257 (Figure 1). We selected ten MAMA PCR positive, fluoroquinolone-resistant and four MAMA PCR negative, fluoroquinolone-sensitive C. jejuni isolates for analysis. The sequence of the 368bp gyrA PCR product of C. jejuni NCTC11168 was used as the reference sequence for the fluoroquinolone-sensitive gyrA. None of the observed changes in the gyrA

548
sequence of fluoroquinolone-sensitive *C. jejuni* isolates corresponded to known changes affecting resistance. However, all of the fluoroquinolone-resistant isolates had a nucleotide sequence change at position 257 as expected.

**MIC and MAMA PCR results were not congruent with respect to *C. coli* fluoroquinolone-resistance**

We also tested the MAMA PCR primers for *C. jejuni* on the 18 *C. coli* samples. A 368bp gyra amplicon was amplified from 10/18 *C. coli* isolates (Table 1). This result was not unexpected as Zirnstein et al. [11] recommended speciation of *Campylobacter* prior to application of the MAMA PCR technique [11]. We confirmed the identity of the *C. coli* isolates using lpxA multiplex PCR (data not shown). The 10 isolates selected were a mixture of fluoroquinolone-resistant (n = 7) and fluoroquinolone-sensitive (n = 3) *C. coli*, with a range of MIC values. Only five of the seven fluoroquinolone-resistant isolates but none of the fluoroquinolone-sensitive *C. coli* isolates were amplified by the *C. jejuni* MAMA PCR method.

DNA sequence analysis of the 368bp gyra partial gene revealed numerous nucleotide differences between *C. coli* and *C. jejuni*. However, all MAMA PCR negative, fluoroquinolone-sensitive isolates (as determined by MIC) had nucleotide sequences comparable to a known fluoroquinolone-sensitive *C. coli* strain (Figure 2). In contrast, all the fluoroquinolone-resistant *C. coli* isolates that amplified a 386bp gyra product had an altered gyra nucleotide sequence at position 256 (C to T). This change was also apparent in the two isolates (C05-20940 and C05-20941) that failed to amplify with the CampyMAMAgyrA5 primer. The partial gyra nucleotide sequence from fluoroquinolone-resistant isolate C05-020774 shared considerable identity to the gyra gene from NCTC11168. The nucleotide sequence encoding the amino acid at position 86 was ATA, identical to the fluoroquinolone-resistance mechanism observed in this study for *C. jejuni*, and different from the ATT sequence in fluoroquinolone-resistant *C. coli* isolates. Based on the nucleotide similarity, lpxA speciation, and multilocus sequence typing results the C05-020774 was MLST type ST1628, typical of *C. coli*.

**Mrp-PFGE analysis demonstrates multiple origins for CIP-resistance in Egypt**

Given the large number of isolates that are fluoroquinolone-resistant in Egypt, we were interested in determining whether this resistance was due to the spread of a single, resistant clone or had arisen in a number of diverse genetic backgrounds. Smal-mrp PFGE profiling demonstrated that fluoroquinolone-resistance has appeared in a variety of diverse genetic backgrounds (Figure 3). This evidence supports the notion that fluoroquinolone-resistance in isolates of *Campylobacter* from Egypt has multiple origins.

**Discussion**

*Campylobacter jejuni* is recognized as an important etiologic agent of human gastrointestinal infection. Fluoroquinolones such as ciprofloxacin are commonly prescribed for empirical treatment of *Campylobacter* enteritis, and therefore resistance to this class of antimicrobial agents is of great concern [19]. Recently, increasing antimicrobial resistance has become a major public health concern in both

**Table 1. Correlation between the carriage of gyra C-257 to T mutation and MIC values of ciprofloxacin and nalidixic acid of *C. jejuni* and *C. coli* isolates.**

<table>
<thead>
<tr>
<th></th>
<th>CIP MIC (ug/ml)</th>
<th>NAL MIC (ug/ml)</th>
<th>gyra (+ or –)</th>
<th>MAMA PCR (+ or –)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) C. jejuni (n=100)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=11</td>
<td>0.032-0.125ug/ml</td>
<td>0.38-3ug/ml</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>n=2</td>
<td>4-12ug/ml</td>
<td>8ug/ml</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>n=87</td>
<td>4-32ug/ml</td>
<td>32-256ug/ml</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>B) C. coli (n=18)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=1</td>
<td>0.047ug/ml</td>
<td>3ug/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n=3</td>
<td>0.047-0.094ug/ml</td>
<td>2-6ug/ml</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>n=7</td>
<td>&gt;32ug/ml</td>
<td>&gt;256ug/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n=5</td>
<td>4-32ug/ml</td>
<td>32-256ug/ml</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>n=2</td>
<td>&gt;32ug/ml</td>
<td>&gt;256ug/ml</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Said et al. - Egypt Campylobacter gyra mutations


---

549
developed and developing countries [3,4,12]. This situation has deteriorated more rapidly in developing countries where there is widespread and uncontrolled use of antimicrobial agents [20]. Published data from Egypt have reported an increase in resistance of both \(C. jejuni\) and \(C. coli\) to fluoroquinolones approaching 39.4% through the years 1995 to 2000 [1,12]. Researchers in Kuwait have reported 50% resistance to CIP among the \(Campylobacter\) spp. studied [21], while countries in Southeast Asia and Spain report that as high as 75-90% of the isolates studied are resistant [19]. Within the USA, fluoroquinolone resistant \(Campylobacter\) in at least one study was as high as 40.5% [22].

Ciprofloxacin inhibits the function of bacterial topoisomerase enzymes, namely, DNA gyrase (bacterial topoisomerase II) and topoisomerase IV [5,23]. Mutations in the gyra gene of Gram-negative bacteria results in resistance to fluoroquinolones by altering the amino acid sequence near the putative active site of the GyrA protein [24].

For \(Campylobacter\) spp., alteration of nucleotide 257 (codon 86) from ACA to ATA in the \(gyrA\) of \(C. jejuni\), and ACT to ATT of the \(gyrA\) of \(C. coli\) has been reported to be the main mechanism of CIP resistance [5,8,19,23,25]. The MAMA PCR technique used in this study allows for the assessment of these nucleotide changes, corresponding to clinically relevant resistance levels to ciprofloxacin, and could be used to rapidly determine whether CIP should be administered therapeutically.
Figure 3. Dendrogram showing the diversity of SmaI-PFGE patterns among *Campylobacter* isolates from Egypt.

Diversity of 107 isolates of *C. jejuni* and *C. coli* is shown after whole genomic digestion using the restriction enzyme SmaI. All samples were normalized in comparison to the CDC molecular weight strain *Salmonella enterica* serotype *Braenderup* strain H9812. Band pattern analysis was performed using Bionumerics v.4.5 and the dendrogram was calculated as described in Materials and Methods. The scale at the top of the figure denotes % similarity (decreasing from right to left). Key: isolate designation, LpxA: the multiplex PCR results, Microbiology: the results of hippurate and IAH tests.
One of the advantages of the MAMA PCR over other techniques that detect alterations in gyrA is the rapid detection of the altered genotype and reliability of results [7-9]. In the present study, the MAMA PCR technique showed 100% sensitivity within the target species, as it was able to detect the point mutation in the 89 C. jejuni resistant isolates, while none of the 11 sensitive isolates were amplified. In most of the isolates, resistance to NA, a quinolone, was observed concomitantly with CIP resistance. However, two of the gyrA, MAMA PCR positive, CIP-resistant isolates, had NA MIC levels of 8 mg/L, considered sensitive by NARMS [15]. DNA sequence analysis confirmed the presence of the C-257 to T mutation as indicated by gyrA MAMA PCR. It is possible that the breakpoint for NA with respect to C. jejuni isolated in Egypt is not identical to that described by NARMS, and may reflect differences in strains and selective pressures between developing and developed countries. Such discrepancies are often observed in the scientific literature reporting Campylobacter susceptibility patterns [26].

We determined the sequence of 14 C. jejuni gyrA PCR products (4 from ciprofloxacin-sensitive and 10 from resistant isolates) to confirm gyrA MAMA PCR and MIC results. In all the resistant isolates tested, the C-257 to T alteration was observed; this change was absent in all the susceptible isolates. Our sequencing results agree with studies conducted elsewhere [26,27]. No other gyrA mutations were observed, notably the GAT-to-AAT (nucleotide position 270; Asp-90 to Asn) [28] or the GCC-to-TCC change (nucleotide position 210; Ala-70-to-Thr) [6]. These results support the conclusion that, in pediatric populations at two different locations in Egypt, the main mechanism of fluoroquinolone resistance is the Thr-86 to Ile alteration in the GyrA protein.

We observed that 10 of 18 C. coli isolates were also amplified by the oligonucleotide primers targeting C. jejuni gyrA gene. MIC values of 7 of the 10 amplified isolates were consistent with CIP resistance, and nucleotide changes at position 257 were confirmed by sequence analysis in all 7 isolates. Only 5 of the 7 resistant isolates were amplified using the MAMA PCR specific primers. Zirnstein [29] showed the identity between the 368bp region of the QRDR of the gyrA gene of C. coli ATCC33559 and the analogous region of C. jejuni ATCC33560 to be 82% with some areas more conserved than others. Thus it is not clear why the C. jejuni primers were able to amplify the C. coli gyrA gene or why in two cases the MAMA PCR primers failed to detect the mutation. In our study, over 50% of the gyrA from C. coli isolates were amplified using these primers. Thus while gyrA MAMA PCR is sensitive for detecting the mutation, without prior speciation, resistance may be incorrectly attributed to isolates of C. jejuni.

We also observed that one C. coli isolate had apparently acquired a segment of the gyrA gene from C. jejuni. The sequence similarity of the QRDR segment of the gyrA gene from C05-020774 to NCTC11168 was greater than 99% (Figure 3). This C. coli isolate apparently has exchanged all or part of its gyrA gene with a fluoroquinolone-resistant C. jejuni isolate. Recombination between C. jejuni and C. coli genomes has been reported before [30,31]. From the sequence analysis, it is not clear why some resistant isolates were able to be amplified using the CampyMAMAgyrA5 primer. Based on these results, we conclude that the MAMA PCR is not specific for C. jejuni gyrA. However, because of the failure of the initial PCR to amplify gyrA from all strains of C. coli, the use of this system for resistance detection in C. coli is not recommended.

Macrolestration profiling using pulsed field gel electrophoresis supports the idea that CIP-resistance has arisen in numerous genetic lineages and is not the result of clonal expansion. Similar findings have been observed in other studies [32]. The implication of this result is that there is a widespread selection pressure on isolates of Campylobacter to become resistant to fluoroquinolones. Whether this pressure is due to agricultural practices or clinical case management has yet to be established. Our results suggest that further studies designed to monitor the prevalence and degree of antibiotic resistance of zoonotic bacteria in animals and humans in Egypt is necessary. These data could contribute to better understanding of the epidemiological links among the isolates from different sources.

Acknowledgements
We express our appreciation to the staff of the Clinical Trials Microbiology Laboratory for their support of this project. Work was supported by work unit# 847705.82000.25GB.E0018. The study protocol was approved by the Naval Medical Research Unit No. 3 Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects. in Research CPHS# 96New DoD #NAMRU-3.2000.0002.

Note
The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as
reflecting the views of the Department of the Navy, Department of Defense, the United States Government, or the Egyptian Ministry of Health and Population.

References

Corresponding author
M. M. Said
US Navy Medical Research Unit -3
PSC 452, Box 5000, FPO AE 09835-0007
Phone: +202-2348-0351; Fax: +202-2342-3428
Email: mayar.said.eg@med.navy.mil

Conflict of interests: No conflict of interests is declared.