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Isolation, genotyping and antimicrobial susceptibility of pathogenic *Escherichia coli* serotypes in ready to eat foods

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**ABSTRACT.** In this study, pathogenic *Escherichia coli* serotypes (*E. coli* O157:H7, O26, O111) and their molecular proximity and antimicrobial susceptibility were investigated in RTE foods. A total of 240 samples; consist of 105 stuffed mussel, 56 meatless cig kofte, 54 Russian salad, 25 cheese halva, were analyzed. The conventional culture and serotyping methods for determination of the organisms were performed and further confirmation by PCR was carried out. Confirmed *E. coli* O157 isolates were genotyped by the enterobacterial repetitive intergenic consensus (ERIC)-PCR. Antibacterial susceptibility testing of the isolates was performed by disc diffusion method. *E. coli* was detected in 7 (2.9 %) of 240 samples, including 3 (5.5%) Russian salad, 3 (2.8%) stuffed mussel, 25 (4 %) cheese halva. Two isolates from Russian salad, 1 from stuffed mussel and 1 from cheese halva were identified as *E. coli* O157. In addition, stuffed mussel isolate was found to carry stx1 and hlyA genes whereas one Russian salad isolate carried the stx1 gene. *E. coli* isolates were found to be resistant to amoxycillin/clavulonic acid, gentamycin and ciprofloxacin, at the rate of 29%, 14% and 29 %, respectively. Only one (14 %) isolate from stuffed mussel was classified as multidrug resistant to three antimicrobials. Furthermore, the isolates, related to O157 and O157:H7, presented different ribotypes in this study. The results provide useful data for the development of public health policy concerning the potential presence of pathogenic antimicrobial resistant *E. coli* serotypes in RTE foods. Strict surveillance of RTE foods at retail points for emerging pathogens, their antimicrobial resistance patterns and the potential likelihood of cross-contamination is required.

**Keywords:** Antimicrobial susceptibility, cheese halva, ERIC-PCR, meatless cig kofte, Russian salad, EHEC, stuffed mussel.

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INTRODUCTION

In recent years, ready to eat (RTE) food consumption has increased because of rapid population growth and the modern lifestyle; longer working hours, increasing women’s participation in the labour market and the change in cooking and eating habits (Tudoran et al., 2012; Oz et al., 2014). RTE foods do not generally require serious pretreatment process and are shelf-stable, delicious, inexpensive and easily accessible to consumers (Spencer, 2005; Jaroni et al., 2010). However, these types of foods present important microbiological risk since they have been implicated as vehicles of food borne microorganisms including Escherichia coli (Ateş et al., 2011; Kochakhani et al, 2016).

E. coli, a member of Enterobacteriaceae family, is the main inhabitant of human and animal guts. They have been accepted as the indicator microorganisms of contamination with fecal and enteric pathogens (Montville et al., 2012). Although most E. coli strains are nonpathogenic, some are known to be responsible for serious human gastrointestinal diseases, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Virulence factors such as shiga toxins (stx1 and stx2), enterohemolysin (hlyA) and intimin (eaeA) play an important role in the pathogenesis of these diseases (Bruyand et al., 2018). Three major surface antigens, O (somatic), H (flagellar) and K (capsule) antigens, are used to serologically to differentiate the E. coli isolates (Montville et al., 2012). Shiga toxin producing E. coli (STEC) strains are the non-O157 E. coli serotypes in RTE foods need to continue in order to complete food safety requires. For this reason, present study aimed to trace the current condition of toxin-producing E. coli contamination in RTE foods based on their prevalence, antimicrobial resistance and phylogenetic relationship.

MATERIALS AND METHODS

The samples of the study were purchased, weekly from January to March 2018, from supermarkets of Nigde and Kayseri cities of Central Anatolia /Turkey. A total of 240 RTE samples including 105 stuffed mussel, 56 meatless cig kofte, 54 Russian salad and 25 cheese halva from fishmongers, meatless cig kofte stores, grocery stores, restaurants and supermarkets (Table 1) were randomly collected. All samples were taken under aseptic conditions and transferred to the laboratory within 2 hours under the cold chain. Mix of stuffed mussels were removed from the shells before analysed.

Reference strains
E. coli O 157 NCTC 12900 (National Collection of Type Cultures 12900) reference strain was used as a positive control for isolation, identification and detection of virulence factors of E. coli O157: H7.

Table 1. RTE food samples

<table>
<thead>
<tr>
<th>RTE food samples</th>
<th>N</th>
<th>Ingredients</th>
<th>Obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stuffed mussel</td>
<td>105</td>
<td>Mytilus galloprovincialis meat with mixed of spices, oil, salt and boiled rice in the cockleshells.</td>
<td>Fishmongers, street venders, cig kofte stores</td>
</tr>
<tr>
<td>Meatless cig kofte</td>
<td>56</td>
<td>Bulgur (pounded wheat) mixed with salt, tomato paste, onions, garlic and spices.</td>
<td>Cig kofte stores</td>
</tr>
<tr>
<td>Russian salad</td>
<td>54</td>
<td>Boiled peas, carrots and potatoes with cucumber pickles mixed in mayonnaise</td>
<td>Restaurants, supermarkets, grocery stores.</td>
</tr>
<tr>
<td>Cheese halva</td>
<td>25</td>
<td>Salt-free fresh cheese is melted and mixed with sugar, flour and semolina on the fire.</td>
<td>Restaurants, supermarkets, grocery stores.</td>
</tr>
</tbody>
</table>
**Bacterial isolation**

A 25 g of each sample was transferred aseptically to 225 mL Trypticase Soy Broth (mTSB, CM129 Oxoid, UK) containing novobiocin (20 g/ml, SR0181E Oxoid, UK) and incubated at 37 °C for 18-24 h. Then, one loopful of enrichment cultures was inoculated onto Chromocult agar (CHROM agar O157, EE222, DRG International, Paris, France) and sorbitol MacConkey Agar (SMAC Agar-109202; Merck KGaA, Darmstadt, Germany) supplemented with 0.05 mg of cefixime and 2.5 mg of tellurite (CT Supplement 109202, Merck KGaA, Darmstadt, Germany). Plates were incubated at 37 °C for 24 h. After incubation, five suspected *E. coli* and *E. coli* O157 colonies were subcultured to blood agar (Oxoid, CM0271) for conducting confirmatory biochemical tests (indole, methyl red, Voges-Proskauer, citrate, urease, sorbitol fermentation and carbohydrate fermentation tests). Subsequently, they were further processed for serological identification (Chapman and Siddons, 1996; Dontorou et al., 2003).

**Serological analysis**

All suspected isolates were tested with *E. coli* O157, *E. coli* H7 antiserum (221591, Difco), and *E. coli* O157 latex agglutination kit (DR0620M, Oxoid) according to the manufacturer’s recommendations.

**DNA extraction**

Total genomic DNA extraction from the isolates was performed using a commercial DNA extraction kit (Axogen Bioscience, Union City, CA, USA) in accordance with the manufacturer’s instructions.

**Confirming *E. coli* isolates**

The universal forward primer targeting the 3′ portion of *trpB* which, together with non-specific *trpA* reverse primer (*trpA2.r*, table 2), yields a 489 bp product from all *E. coli* strains was included in the reaction as an internal control as mentioned by Clermont et al. (2008).

**PCR analysis for the detection of fliC*, rfbO111, wzx-wzyO26 and rfbO157 genes**

The primer pairs for *fliC*, *rfbO157*, *wzx-wzyO26* and *rfbO111* genes and the PCR assay conditions were performed in reference to Sarimehmetoglu et al. (2009), Maurer et al. (1999), Paton and Paton (1998) and Durso et al. (2005), respectively.

| Table 2. Primers and PCR amplification products used in this study  |
|------------------|------------------|------------------|------------------|
| PCR Reaction     | Target gene      | Primer           | Sequence (5′-to 3′) | Size of PCR ampl. (bp) | Reference |
| Internal control | *trpB*           | *trpB*.f         | CGGCGATAAAGACATCTTC | 489                  | Clermont et al. (2008) |
|                  | *trpA*           | *trpA*.r         | GGACGGGCGGCTGGCAGAG | 625                  | Sarimehmetoglu et al (2009) |
| H7               | *fliC*           | *fliC*.F         | GTGCTGAGTTGATTTTGC | 420                  | Maurer et al. (1999) |
|                  |                 | *fliC*.R         | AGATGTTGACCTACCGTC |                      |          |
| LPS O157         | *rfbO111*        | *rfbO111*.F      | TAGAGAAATATCAAAGTATCC | 406                  | Paton and Paton (1998) |
|                  |                 | *rfbO111*.R      | ATACGGTTGACACATCTTCC |                      |          |
| O111             | *wzx-wzyO26*     | *wzx-wzyO26*.F   | AAATAGAAGCGCTTCATGC | 596                  | Durso et al. (2005) |
|                  |                 | *wzx-wzyO26*.R   | CCAGCGAAGCACAATATGAC |                      |          |
| Shiga-like toxin 1| *stx1*           | *stx1*.F         | TGGTTACGAAAGGTTTGGTT | 210                  | Fratamico et al. (2000) |
|                  |                 | *stx1*.R         | GCTATCTGATCAACAAAGAATAC |                      |          |
| Shiga-like toxin 2| *stx2*           | *stx2*.F         | GTTTTTTCTCGAGTATCTTTC | 484                  | Fratamico et al. (2000) |
|                  |                 | *stx2*.R         | GATGCATCTCGTATCTTAC |                      |          |
| Intimin          | *eaeA*           | *eaeA*.F         | ATTACTCAACACGACACGTT | 397                  | Fratamico et al. (2000) |
|                  |                 | *eaeA*.R         | ACAGCGTTGAGGTAACACCT |                      |          |
| Enterohemolysin  | *hlyA*           | *hlyA*.F         | ACAGATGTTGATTTTTCGGA | 166                  | Fratamico et al. (2000) |
|                  |                 | *hlyA*.R         | CTTCAGTCACCATACTAT |                      |          |
Detection of virulence genes (stx1, stx2, eaeA and hlyA) by Multiplex PCR

Multiplex PCR (mPCR) targeting virulence genes of E. coli O157: H7, comprising stx1, stx2, eaeA and hlyA (Table 2) was carried out in a study conducted by Fratamico et al. (2000).

Electrophoresis of all amplified products was carried out in 1.5% agarose gel containing 0.06% ethidium bromide for 50 minutes at 100 V (EC250-90, Thermo, Pittsburgh, Pa., USA) and visualized on a U.V transilluminator (Vilber Lourmat, Marne La Vallee, France).

ERIC-PCR

The ERIC-PCR was carried out on four isolates identified as EHEC. The total 50 µL of PCR mixture prepared including of 1xPCR buffer (Vivantis, Chino, CA, USA), 0.2 Mm dNTP mix (Vivantis), 4 mM MgCl₂ (Vivantis), 5 U Taq polymerase (Vivantis), 25 pmol each primer and 1and 1µL target DNA. ERIC-PCR was performed under the following conditions: initial denaturation at 94 °C for 5 min, 94 °C for 1 min, 25 °C for 1 min, and 72 °C for 2 min (Techne TC-512, Keison Products, Chelmsford, Essex, UK). The amplified product were subjected to electrophoresis at 100 V for 1h on 2 % agarose gel and was monitored by visual inspection under UV light for distinct DNA profiles (Houf et al., 2002). Banding patterns were photographed and analysed by scoring presence (1) or absence (0) of bands for prediction of similarity. Dendrogram was made by construction of a phylogenetic tree using the online software dendrogram construction utility, DendroUPGMA (http://genomes.urv.cat/UPGMA) (Garcia-Vallvé and Puigbo, 2002).

Antimicrobial susceptibility

Antimicrobial susceptibility of all E. coli isolates were tested using disk diffusion methods for Amoxicillin/Clavulanic acid (AMC) (30 µg), Ciprofloxacin (CIP) (5 µg), Gentamicin (GEN) (10 µg), Meropenem (MER) (10 µg) and Trimethoprim/ sulfamethoxazole (STX) (25 µg) according to EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing. Clinical breakpoint tables v. 8.1; http://www.eucast.org v.8.1, accessed: 12.08.2018).

Table 3. Results for pathogenic E. coli serotypes, their virulence genes from RTE foods

<table>
<thead>
<tr>
<th>RTE food samples</th>
<th>N</th>
<th>n(%)</th>
<th>rfbO157, fliC7, rfbO111 and wzx-wzyO26 and virulence genes</th>
<th>Zone of inhibition (mm) in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>rfbO157 fliC7 rfbO111 wzx-wzyO26 stx1 stx2 hlyA eaeA</td>
<td>Zone of inhibition (mm) in this study</td>
</tr>
<tr>
<td>Stuffed mussel</td>
<td>105</td>
<td>3 (2.8%)</td>
<td>1 (0.95%) 1 (0.95%) - - 1 (0.95%) 1 (0.95%) -</td>
<td></td>
</tr>
<tr>
<td>Cig kofte</td>
<td>56</td>
<td>-</td>
<td>- - - - - - -</td>
<td></td>
</tr>
<tr>
<td>Russian salad</td>
<td>54</td>
<td>3 (5.5%)</td>
<td>2 (3.7%) - - - 1 (1.85%) - - -</td>
<td></td>
</tr>
<tr>
<td>Cheese halva</td>
<td>25</td>
<td>1 (4%)</td>
<td>1 (4%) 1 (4%) - - - - -</td>
<td></td>
</tr>
</tbody>
</table>

n: Detected E. coli by trpA gene

Table 4. Antimicrobial susceptibility profiles of E. coli isolates

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Diameter of the inhibition zones of E. coli according to EUCAST, 2018 (mm)</th>
<th>Zone of inhibition (mm) in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S≥</td>
<td>R&lt;</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid (AMC)</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>Gentamicin (GEN)</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Meropenem (MER)</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>Trimethoprim/ sulfamethoxazole (STX)</td>
<td>14</td>
<td>11</td>
</tr>
</tbody>
</table>

S: Susceptible, R: Resistant
RESULTS
Seven (2.9%) out of 240 RTE samples were found positive as a result of conventional culture methods and were confirmed by PCR. Furthermore, of the 7 E. coli isolates, 2 (3.7%) from Russian salad were identified as E. coli O157 based on PCR and serotyping and 1 (1.85%) of them found to carry stx1 gene. E. coli O157:H7 was detected in 2 (0.83%) out of 240 samples including 1 (0.95%) stuffed mussel and 1 (4%) cheese halva. One isolate from stuffed mussel were found to harbour the stx1 and hlyA genes. However, E. coli O111 and O26 were not detected in any sample.

The results of antibiotic susceptibility test have been summarized in Table 4. All isolates of E. coli were highly sensitive to MER and STX. Resistance to AMC occurred in 2 (29%) E. coli isolates from stuffed mussel, one of which was multidrug resistant to three antibiotics (AMC, CIP and GEN). Furthermore, stx1 gene carrying E. coli O157 isolate obtained from Russian salad was found to be resistant to CIP.

Figure 2 resumes the ERIC-PCR profiles of pathogenic E. coli serotypes. ERIC-PCR genotyping revealed 7-18 fragments resolved per isolate. All of 4 pathogenic E. coli isolates under analysis produced 3-7 amplicons ranging from 150 to 1500 bp. Phylogenetic tree (Fig. 2) showed that highly polymorphic DNA fragments among the 4 pathogenic E. coli isolates. The Jaccard similarity coefficient of the genotypes was ranging from 0.143 [(A (O157 serotype, carried stx1 gene, from Russian salad) and B (O157 serotype from Russian salad)] to 0.125 [B and C (O157 H7 serotype from stuffed mussel)].

DISCUSSION
The RTE foods, frequently preferred by the consumers in recent years, are pre-cooked or prepared and packaged with a suitable material and often require minimal preparation (Spencer, 2005). Wide range of RTE foods, that can be bought from markets, street vendors, restaurants and stores, may contain a variety of microorganisms, while many of which are harmless, some are dangerous (Elobeid et al. 2014; Jaroni et al., 2008). In this study, pathogenic E. coli serotypes (E. coli O157:H7, O26, O111) was carried out from RTE foods in Central Anatolia region. The content of RTE foods examined in the study are raw and cooked materials, plants, cheese and shellfish with high protein, spices and sauces (Table 1).

Stuffed mussel is a highly consumed traditional shellfish in Turkey. Reported results demonstrated that 3 of 105 (2.85%) stuffed mussel were found to...
be positive for E. coli and one of them was defined as O157 H7 (0.95%) containing stx1 and hlyA genes. It was found that one E. coli strain was resistant to three antibiotics (AMC, CIP and CN); other E. coli strain was resistant to only AMC. Studies on the microbiological quality of stuffed mussels in Turkey demonstrated that they may be contaminated with some foodborne pathogens including E. coli however no investigation is available on pathogenic E. coli serotypes in stuffed mussels samples (Bingol et al., 2008; Hampikyan et al., 2008; Ateş et al., 2011; Koçatepe et al., 2016). Similar to our results Surendraraj et al. (2010) in India also reported 8.3 % of shrimp samples were contaminated with EHEC isolates which were positive for eaeA, stx and hlyA genes with low incidence of multiple antibiotic resistance. Prakasan et al. (2018) recently reported 33.33% of shellfish samples were contaminated with Shiga toxin-producing E. coli. *Mytilus galloprovincialis* is a filter feeder organism which collects pathogenic microorganisms and different harmful residues including heavy metals and agricultural waste, as well as organic materials from the coastal and estuarine environments. In addition, high amino acid content, high pH (approximately 6.55) and high water activity (0.98) of mussels facilitate to colonization and transmission of E. coli and other pathogens (Sengor et al., 2004; Gourmelon et al. 2006). However preparation of the stuffed mussels includes cooking period that is high enough to kill most vegetative cells (Kisla ve Uzgun, 2008). According to Kisla ve Uzgun (2008), stuffed mussels were commonly exposed to unsuitable environmental conditions such as soil, dust, insects, flies etc and high ambient temperatures during retail sale for long times. We also collected stuffed mussel samples from fishmongers which was an outside sale under unsuitable environmental conditions. Furthermore, stuffed mussel mix (spices, oil, salt and boiled rice) is stuffed with hand in the cockleshells (Ates et al., 2011). E. coli is classified as faecal coliform and presence of this bacteria in the samples may indicate errors and omissions in handling, lack of sanitary practices by foodhandlers and possible cross-contaminations.

In this study, E. coli O157:H7 was isolated from only 1 of 25 (4 %) cheese halva samples. According to literature screening, there is no research related to E. coli O157:H7 in cheese halva in Turkey. Nevertheless Secim et al. (2017) investigated presence of E. coli in cheese halva samples and reported no contamination. The presence of E.coli has been investigated in cheese desserts in some studies; Cokal et al. (2012) and Secim et al (2017) reported that no E. coli contamination in Hosmerim desserts. The significance of E. coli O157:H7 contamination in milk and cheese samples has previously been reviewed (Zweifel et al., 2010; Lynch et al., 2012). As the cheese halva is a heat-treated dessert, the presence of E. coli O157:H7 in cheese halva might have originated from post heating contamination during packaging process or personnel. Although E. coli is inactivated by some barrier factors like heat treatment in the processed foods, subsequent cross contamination could be of concern (Wahi et al., 2006).

In the present study, 3 Russian salad samples (5.5%) were found positive for E. coli, 2 of which (3.7 %) were determined as E. coli O157 with stx1 gene and CIP resistance was detected in one of them. Russian salad is a mayonnaise based salad. Although mayonnaise is relatively resistant to microbial spoilage due to its low pH, it is known that E. coli and pathogenic E. coli serotypes have inducible acid resistance mechanisms. A study by Zhao and Doyle (1993) revealed that E. coli O157:H7 can survive at 5°C in mayonnaise for several weeks, in case of unsuitable manufacturing practices or any type of cross-contamination (contaminated vegetables in salad, dirty kitchen equipments, food handlers etc) of mayonnaise. In this study, Russian salad samples were bought from restaurants and grocery stores in which ready to eat foods were sold at retail without package. The contamination may be associated with unhygienic ingredients of salad, food handlers, utensils and contact surfaces.

In our study, no E. coli or pathogenic E. coli serotypes was detected in meatless cig köfte samples. Although meatless cig köfte can serve as a vector for the transmission of some human pathogens (Taban, 2012; Delikanli et al. 2014), no reports are available about the examination of E. coli O157:H7 in meatless cig köfte samples. Several studies have demonstrated that garlic, spices and onion which are meatless cig köfte ingredients are able to inhibit pathogenic E. coli serotype growth, depending on the concentration, storage time and temperature (Koidis et al., 2000; Kim and Kim, 2007; Rounds et al., 2013).

In this study, one isolate found to carry stx1 and one isolate hlyA gene. These results for detection rates of toxin genes were higher than the study conducted by Cho et al. (2010) which showed absence of the stx genes of street-vended foods in Korea. However, Gupta el al. (2012) reported from India the previ-
lence of stxl and stx2 genes of RTE fish product were 5.5% and 7.4% respectively, higher than our results. The pathogenicity of E. coli serotypes are related to their virulence factors, shiga toxins, enterohemolysin and intimin. Enterohemolysin (encoded by the hlyA gene) causes the lysis of erythrocytes, which provide iron uptake in the intestinal environment (Dontorou 2003). Shiga toxins (Stx 1, 1c, 2, 2c, 2d, 2dact, 2e, 2f) are the primary virulence factor of pathogenic E. coli serotypes which can be defined as the locus enterocyte effacement (LEE) of the adherence system (Obrig 2010). Stx lead to inflammatory and thrombogenic changes in the endothelial cells causing HUS and thrombotic microangiopathy (TMA), especially effects kidneys and other potential organs (Bruyand et al., 2018). E. coli O111 and O26 were not detected in any sample in our study. In contrast, the current results were reported by Hassanin et al. (2014), for RTE meat and chicken products, the rates of O111 and O26 serotypes were between 6.7-33.3%.

Results of this study demonstrated that MER and STX were the most effective agents against E. coli with susceptibility rate of 100%. Recent studies have also been describing STX and MER resistant E. coli isolates (Campos et al., 2013; Rasheed et al., 2014; Lima et al. 2017; Ye et al., 2018) in RTE foods. Of the 7 E. coli isolates examined, we found an overall prevalence of 42% (n=3) isolates showed resistance rate to AMC (29%), CIP (29%) and GEN (14%) (Table 4). AMC, CIP and GEN resistance has been reported in studies performed worldwide, concerning RTE foods (Rasheed et al. 2014; Kochakkhani et al. 2016; Baloch et al. 2017; Ye et al., 2018). Our results were lower than those found by Kochakkhani et al. (2016) (100% for AMC and CIP) and Baloch et al. (2017) (80.9% for AMC and 18% for GEN). Also, the low percentage of resistance to AMC, CIP and GEN was noted by Campos et al. (2013) (5% for CIP), Lima et al (2017) (none for CIP and GEN) and Rasheed et al. (2014) (8.6% for AMC). Moreover 1 (14.2%) multidrug resistant isolate also detected in the study (Table 4). This result is in accordance with those reported by Lima et al. (2017) and Baloch et al. (2017) as 13.3% and 17.6% resistance rate respectively. The existence of multidrug resistant strain could create serious threat to the patients because of transferring antimicrobial resistance genes to other pathogens and to humans through food.

The prevalence of pathogenic E. coli serotypes always should be carefully evaluated in RTE foods. To our knowledge, no study concerning the prevalence of pathogenic E. coli serotypes in RTE foods, including the detection of virulence genes, genotyping and antimicrobial susceptibility, has been conducted previously in Turkey. Results of the study would be useful for monitoring of pathogenic, antibiotic resistant E. coli serotypes and for providing information about possible role of RTE foods acting as a vehicle for this pathogen.

CONFLICT OF INTEREST
None declared by the authors.

REFERENCES


