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Title	Identification of Escherichia coli strains from water vending machines of Kelantan, Malaysia using 16S rRNA gene sequence analysis
Type	Article
URL	https://clock.uclan.ac.uk/id/eprint/14033/
DOI	https://doi.org/10.1007/s12403-016-0194-x
Date	2016
Citation	Tan, Ee Yau, Arifullah, Mohamad and Soon, Jan Mei (2016) Identification of Escherichia coli strains from water vending machines of Kelantan, Malaysia using 16S rRNA gene sequence analysis. Exposure and Health. ISSN 1876-1658
Creators	Tan, Ee Yau, Arifullah, Mohamad and Soon, Jan Mei

It is advisable to refer to the publisher's version if you intend to cite from the work.
<https://doi.org/10.1007/s12403-016-0194-x>

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1 **Identification of *Escherichia coli* strains from water vending machines of Kelantan,**
2 **Malaysia using 16S rRNA gene sequence analysis**

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10
11
12 **Abstract**

13
14 Water vending machines provide an alternative source of clean and safe drinking water to the
15 consumers. However, the quality of drinking water may alter due to contamination from lack of
16 hygienic practices and maintenance of the machines. Hence this study was conducted to determine
17 the microbiological quality of water from vending machines and associated contact surfaces.
18 Seventeen water samples and 85 swab samples (nozzles, drip trays, coin slots, buttons and doors)
19 from 3 locations in Kelantan were collected. Polymerase Chain Reaction (PCR) amplification and
20 16S ribosomal ribonucleic acid (rRNA) sequencing were carried out and sequences obtained were
21 compared against the sequences available in the National Centre for Biotechnology Information
22 (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program. Coliform
23 counts were observed in 94.12% of water samples, 76.47% of nozzles and 82.35% of drip tray
24 swabs. Furthermore, results of 16S rRNA sequence analysis indicated that two gram-negative
25 isolates were identified as *Escherichia coli* U 5/41 (Accession no. NR_024570.1) and *Escherichia*
26 *coli* O157:H7 EDL933 (Accession no. CP008957.1) with similarity value of 100% respectively.
27 The results from this study further improve our understanding of the potential microorganisms in
28 drinking water. Regular maintenance and cleaning of water vending machines are important to
29 reduce bacterial growth and presence of waterborne pathogens.

30
31 **Keywords:** coliform; drinking water; *Escherichia coli*; Polymerase Chain Reaction

32
33 **Introduction**

34
35 A water vending machine (WVM) is an automated self-service machine which dispenses water
36 into the container when sufficient coins, bills or tokens are inserted (Price et al. 2006). Most
37 freestanding floor models of WVM are located at locations such as outside grocery stores,
38 supermarkets, or retail outlets. Access to reverse osmosis (RO), drinking water in vending
39 machines (VMs) can improve quality of water in terms of organic, inorganic and bacteria content.

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40 RO can retain 99% of bacterial cell on the membrane, leaving less than 50 cell/ml in drinking
41 water (EPA 2011; Ladner 2009).

42
43 Although well-designed WVMs are established and provided water treatments via RO, carbon
44 filtration and UV radiation, there are still possibilities for microbe to be transmitted to water
45 dispensers. Coliform bacteria can colonize the carbon filters of WVM resulting in high
46 concentration of coliform bacteria in the final vended water. Suppliers or service operators of VMs
47 need to examine the quality and safety of the water from VMs. Sampling and analyses of the
48 vended water for bacteriological quality should be conducted to ensure public safety. Continuous
49 monitoring of water quality from VMs and distribution parts of the VMs are essential to meet the
50 quality requirements of ISO (WHO 2004).

51
52 However, the quality of water from VM may rapidly alter as a response to alteration in the
53 surrounding environment of the VMs (Ali et al. 2012). Poor safety and hygiene practices when
54 handling vending machines may transport pathogenic organisms and toxic chemicals to
55 community which causes harm to consumers. Water contamination caused by poor sanitation and
56 hygiene and water quality is among the top ten prevalent water-borne diseases in developing
57 countries (Prasai et al. 2007). Inappropriate cleaning and contamination of the WVM's nozzles
58 may result in biofilm formation and bacteria survival. According to Bloomfield et al. (2012), some
59 heterotrophic bacteria such as *Pseudomonas aeruginosa* can adhere to the surface of WVMs such
60 as buttons to form biofilms. Dispenser or nozzles of VMs may be contaminated with heterotrophic
61 bacteria. Therefore, drinking water from VMs must be suitable for consumption and free from
62 pathogenic microorganisms to ensure public safety. Hence the aim of this study was to determine
63 the microbiological quality of water from VMs and associated contact surfaces.

64 65 **Materials and Methods**

66
67 Sterile Schott Duran bottles containing 2 ml of sterile 10% sodium thiosulfate were used to collect
68 samples for microbiological analyses. Triplicate samples of 17 WVM from three locations in
69 Kelantan (Jeli town, Tanah Merah town and an institution of higher learning in Jeli) were collected,
70 kept in ice box containing crushed ice and transported back to the laboratory for microbiological
71 analysis. Temperature of water samples were taken at the water vending machine sites. Each water
72 samples (100 ml) were labelled with date and time of collection and site collection. Sterile cotton-
73 tipped swabs were used to swab surfaces of dispense nozzles, drip trays, vending machine buttons,
74 trap doors, coin receiving and dispensing slots. The sterile cotton-tipped swabs were dipped in
75 sterile test tube containing 2 ml of sterile neutralizer, transferred to Whirl-Pak sampling bags, kept
76 in ice box and transported to the laboratory. Water and swab samples collection were carried out
77 according to APHA (1998) and Shar et al. (2008).

78
79 *Sample preparation and serial dilution*

80
81 A total of 1ml of water samples were added into the test tube that contains 9ml of buffered dilution
82 water. Diluted water samples from 10^{-1} to 10^{-4} were prepared aseptically for aerobic plate counts,
83 coliform and *E. coli* tests.

84
85 *Coliform and E. coli test*

86
87 Total coliform were enumerated using multiple tube fermentation technique. Complete and
88 positive coliform test were streaked on sterile Eosin Methylene Blue (EMB) agar using spread
89 plate method and incubated at 37°C for 24 hours. Green metallic colonies were recognised as
90 *Esherichia coli* and subjected to biochemical tests (methyl red, citrate and indole tests). Isolated
91 bacteria from positive biochemical tests were selected and streaked on nutrient agar. The
92 morphology characteristics of isolated bacteria were observed. In order to identify the bacterial
93 strains, genomic DNA extraction, amplification and 16S rRNA sequence analysis was carried out.

94
95 *Microbial culture and DNA extraction*

96
97 Isolated *E. coli* colony on nutrient agar were inoculated into 10 ml trypticase soy broth (TSB) and
98 incubated overnight at 37°C in an orbital shaker at 150 rpm. Bacterial cultures were pelleted down,
99 when the OD of culture reached to 0.8-1.0 at 600nm. DNA extraction was conducted to obtain the
100 genomic DNA fragment of isolated bacteria from vending machines no. 5 and no.12 for PCR and
101 16S rRNA analysis. DNA extraction was conducted using G-spin Total Genomic DNA Kit (Intron,
102 Korea).

103
104 *Polymerase Chain Reaction and 16S rDNA sequence*

105
106 Universal primers (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-
107 CTTGTGCGGGCCCCGTC AATTC-3') were used for the amplification of the 16S rDNA gene
108 fragment. PCR reaction was carried out in 50 µl reaction mixture containing: 10 ng of genomic
109 DNA, 2.5 U of Taq polymerase, 5µl of 10X PCR amplification buffer (100 mM Tris-HCL, 500
110 mM KC1 pH 8.3), 200µM dNTP, 10 p moles each of the universal primers and 1.5 Mm MgCl₂.

111
112 Reaction was in a programmable thermal cycler (Eppendorf AG 22331 Hamburg, Germany) and
113 the program included an initial denaturation at 94°C for 3 minutes and then 30 cycles of
114 denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min, with a
115 final extension for 10 min at 72°C. A 6 µl of PCR product was subjected to 1% agarose gel
116 electrophoresis for 45 min at 80 V. Gels were stained with ethidium bromide and PCR products
117 were visualized using a UV transilluminator and photographed. PCR products were purified using
118 QIAquick PCR purification kit (Qiagen, Germany) and sent to First BASE Laboratories Sdn. Bhd.,
119 Malaysia for sequencing.. The sequences obtained were compared against the sequences available
120 in the National Centre for Biotechnology Information (NCBI) database using BLAST program.

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Results and Discussion

Analysis of water samples

Out of 17 samples tested, 94% of coliform bacteria were observed in 16 samples (Fig. 1). Similar results were reported by Hertin (2011), when beverage samples dispersed from 18 soda fountain machines contained 86% of coliform bacteria and exceeded the EU standard for drinking water. This may be due to insufficient cleaning and sanitation of the WVMS. Low quality of membrane filtration and lack of disinfection may contribute to bacteria re-growth after water treatment. High coliform bacteria present in drinking water also indicate that the water treatment system in VMs are not being sanitized and maintained on a regular basis. This is in agreement with Tobin et al. (1981) who mentioned that lack of maintenance on carbon filter of the vending machines may further contaminate drinking water from VMs. Poor machines condition such as missing door also increase chances of contamination of water. A study conducted by Du and Knorr (2004) reported that contamination of drinking water were attributed by poor cleanliness and maintenance services provided by the VMs owners.

Nozzles, drip trays and door swabs

A total of 13 out of 17 nozzle samples (76.47 %) were positive for coliform (Fig. 1). Nozzles were also found to contain the highest coliform count compared to other contact surfaces. According to Robertson (1987) and Lakshmanan and Schaffner (2006), nozzles may be the most soiled areas of the VMs as small volume of water still remains in the nozzle after dispensing.

14 out of 17 tray swab samples (82.35 %) were positive for coliform (>2 Most Probable Number [MPN]/100ml). Drip tray from VM no. 12 recorded the highest coliform count (> 1600 MPN/100ml). On the other hand, trays from VMs no. 10, 11, 16 and 17 showed negative results for coliform (<2 MPN/100ml). The service intervals conducted by operators were shorter for VMs no. 10, 11 and 17. Good cleaning services can minimize bacteria growth on the tray of WVM. Tray contamination could also occur when dirty bottles were placed on the tray. Eleven samples out of 17 (64.70 %) door swab samples were contaminated with coliform. Door swab sample no.12 has the highest coliform bacteria (900 MPN/100ml). The major causes of door contamination may be due to human contact by consumers with poor personal hygiene (Elalfy 2007).

Coin slots and button swabs

6 out of 17 coin slot and button swabs (35.29 %) were positive for coliform. The results indicate that both the coin slots and button swab samples have low coliform counts and do not contribute

160 significant contamination to the VMs. The dry environment of the coin slots and buttons may
161 have suppressed the growth of microorganisms.

162

163 **Fig. 1** Coliform count (MPN/100 ml) of water and associated surfaces of vending machines

164

165 *Physico-chemical analyses*

166

167 Turbidity ranged between 0.22 and 3.48 Nephelometric Turbidity Unit (NTU) with a mean
168 turbidity of 1.06 NTU. When turbidity level exceeds 1 NTU, there is high possibility that
169 microorganisms will be present in the water due to increased protection from disinfectant.
170 Environmental Protection Agency (EPA, 2012) drinking water standard stated that critical
171 acceptance level for turbidity should be between 0.5 - 1.0 NTU while IBWA (2015) stated that the
172 turbidity in drinking water shall not exceed 0.5 NTU. Based on Table 2, 56.25% water samples
173 exceeded the EPA drinking water standard. The highest turbidity value in water sample was found
174 in VM no. 12 with a mean turbidity value of 3.48 ± 0.23 NTU (Table 1).

175

176 Turbidity level can be used to indicate the cloudiness of water dispensed from WVMs. High
177 turbidity value indicates lower quality of drinking water. High turbidity is associated with higher
178 amount of organic and particles in the water. This might protect pathogenic microorganisms
179 (which are encased in the particles) against disinfection in WVMs (Rim et al. 2009). High turbidity
180 value in drinking water also may be due to the presence of dust and biofilm in nozzles of WVM
181 (Chaidez et al. 2010).

182

183 According to Ali et al. (2012), high turbidity level in drinking water may lead to illnesses such as
184 diarrhoea and vomiting. Turbidity is a quality control parameter and can be used as an alert for
185 operators in order to ensure effectiveness of water treatments. High total aerobic count is also
186 associated with higher levels of turbidity which may have potential to cause illness. This can be
187 shown in water sample no. 12 which has high turbidity and the highest concentration of coliform
188 bacteria (>1600 MPN/100ml).

189

190 WHO and EPA recommended the pH value for drinking water should ranged from 6.5 to 8.50 (Ali
191 et al. 2012). In this study, the water samples ranged between pH 6.23 and 8.75. This indicates
192 11.76% water samples exceeded the limit of acceptance of WHO and EPA. Water sample from
193 VM no. 12 exceeded the limit of recommendation with a pH value of 8.75. pH of water outside
194 the recommended range will have undesirable effects in terms of taste and odour (Mako et al.
195 2014). Poor management of membrane filtration may alter the pH of the water dispensed from
196 WVMs.

197

198 **Table 1** Physico-chemical results of water from vending machines (n=17)

199

200 *Identification of bacterial strains*

201
202 In total, 6 isolates were selected from positive EMB plates and biochemical tests (methyl red test,
203 citrate test and indole test) and subcultured on nutrient agar to obtain pure culture. Basic
204 identification of pure culture from NA was conducted to analyze the basic morphology of bacteria
205 such as shape, nature of axis and staining colour. The morphological characteristics of isolated
206 bacteria was summarised in Table 2. *E. coli* could be identified as circular, raised, with entire
207 margin, opaque, small and non-endospores forming rod (State et al. 2008). Based on the
208 morphological characteristics and reddish pink colour (Gram negative) from the Gram staining
209 procedure, isolates from VM no. 5 and 12 were selected for 16S rRNA analysis.

210
211 **Table 2** Morphological characteristics of isolated bacterial colony

212
213
214 *Polymerase chain reaction*

215
216 Genomic DNA of E2 and E6 were used in polymerase chain reaction (PCR) in thermal cyclor. The
217 purpose of PCR is to amplify the targeted region in *E. coli* from water sample and maximize
218 selectivity for *E. coli* (Pupo et al. 1997; Sabat et al. 2000). 1 Kb ladder (Vivantis) was used to
219 estimate molecular weight of PCR product. Based on Fig. 2 , a single and clear band of 1500 bp
220 of 16S rRNA fragment was observed in lane 2 and 6 on agarose gel under UV light. This indicates
221 that fragment of genomic DNA of *E. coli* was successfully amplified by the used primers 27F
222 and 1492R which were properly bound to specific sites of the DNA template during primer
223 annealing (Ramadan et al. 2015). The findings are similar to a study reported by Momba (2012),
224 where all amplified PCR products from groundwater samples containing pathogenic
225 microorganisms appear as single band of 1500bp under UV light. Sterilized nucleus free water was
226 used in negative control instead of DNA products.

227
228 **Fig. 2** PCR product after gel electrophoresis on 1.0% agarose gel



230 *PCR purification*

231

232 PCR products were purified with the QIAquick spin column to remove residual reagents used
233 in the thermal cycler. Before sending purified product to First Base Laboratories Sdn. Bhd.,
234 Malaysia for DNA sequencing, agarose gel electrophoresis was used to confirm the presence
235 of band inside the PCR products. The products were further subjected to DNA sequencing with
236 the origin primers 27F and 1492R to identify strains of *E. coli*.

237

238 After receiving the DNA sequencing result from First Base Laboratories PLC, two isolates
239 were aligned by using BLAST analysis and the identified *E. coli* strains are shown in BLAST
240 is used to analyze the alignment by matching up each position of 16S rRNA gene sequences to
241 each position of the sequences in the database. The percentage of similarity of isolated sample
242 was compared with the geneBank sequence. The 16S rRNA gene from VMs no. 5 and no. 12
243 have been identified as *Escherichia coli* strain U 5/41 and *Escherichia coli* O157:H7 str.
244 EDL933 with similarity value of 100% respectively.

245

246 According to Public Health England (n.d.), *Escherichia coli* U 5/41 is classified as hazard
247 group 2 which is likely to cause human diseases. Presence of *Escherichia coli* O157:H7 str.
248 EDL933 in water samples could be linked with biofilm formation. Biofilm formation is one of
249 the sources that contribute to diseases in relation with public health (Beloin et al. 2008; Parsek
250 and Singh 2003). Meanwhile, *Escherichia coli* O157:H7 is the major cause of haemorrhagic
251 colitis and haemolytic uremic syndrome (HUS) (Andreoli et al. 2002). *Escherichia coli*
252 O157:H7 can lead to outbreak of gastrointestinal diseases including bloody diarrhoea, kidney
253 failure, abdominal cramps even severe hemorrhagic colitis (Peacock et al. 2001).

254

255 **Conclusion**

256

257 In this study, 16s rRNA sequencing identified two bacterial strains isolated from drinking water
258 from VMs of Kelantan, Malaysia as *Escherichia coli* U 5/41 and *Escherichia coli* O157:H7 str.
259 EDL933. The presence of pathogenic *E. coli* in drinking water poses potential threat to humans
260 consuming the water. Regular maintenance, cleaning and sanitation of WVMs should be
261 carried out and consumers should be educated about good personal hygiene practices to prevent
262 cross contamination (i.e. dirty water containers in contact with drip trays, dirty hands in contact
263 with buttons).

264

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370 **Table 1** Physico-chemical results of water from vending machines (n=17)
371

Vending Machines	Location	Mean Turbidity (NTU)	Mean Temperature (°C)	Mean pH
1		0.65±0.04	30.5±0.00	6.94±0.04
2		0.22±0.01	29.0±0.00	6.94±0.06
3		0.45±0.00	30.0±0.00	6.73±0.04
4	Institute of higher learning	0.36±0.01	31.0±0.00	6.23±0.03
5		1.03±0.29	27.0±0.00	8.21±0.02
6		1.02±0.20	29.0±0.00	6.81±0.02
7		0.31±0.03	27.0±0.00	6.89±0.01
8		0.40±0.02	28.0±0.00	7.17±0.03
9		1.53±0.37	29.0±0.00	6.84±0.02
10	Jeli	1.67±0.26	32.0±0.00	6.84±0.01
11		1.92±0.18	31.0±0.00	7.33±0.03
12	Tanah Merah	3.48±0.23	27.9±0.00 ^a	8.75±0.01
13		1.74±0.32	28.0±0.00 ^b	6.90±0.01
14		0.76±0.21	30.0±0.00 ^d	6.84±0.02
15	Jeli	1.14±0.05	32.0±0.00 ^f	6.79±0.03
16		0.36±0.08	31.0±0.00 ^e	7.31±0.01
17	Tanah Merah	1.06±0.24	31.0±0.00 ^e	7.35±0.00
Average		1.06±0.83	29.6±0.00	7.11±2.55

Different superscript letters (a-e) in the same column indicate significant difference (p<0.05)

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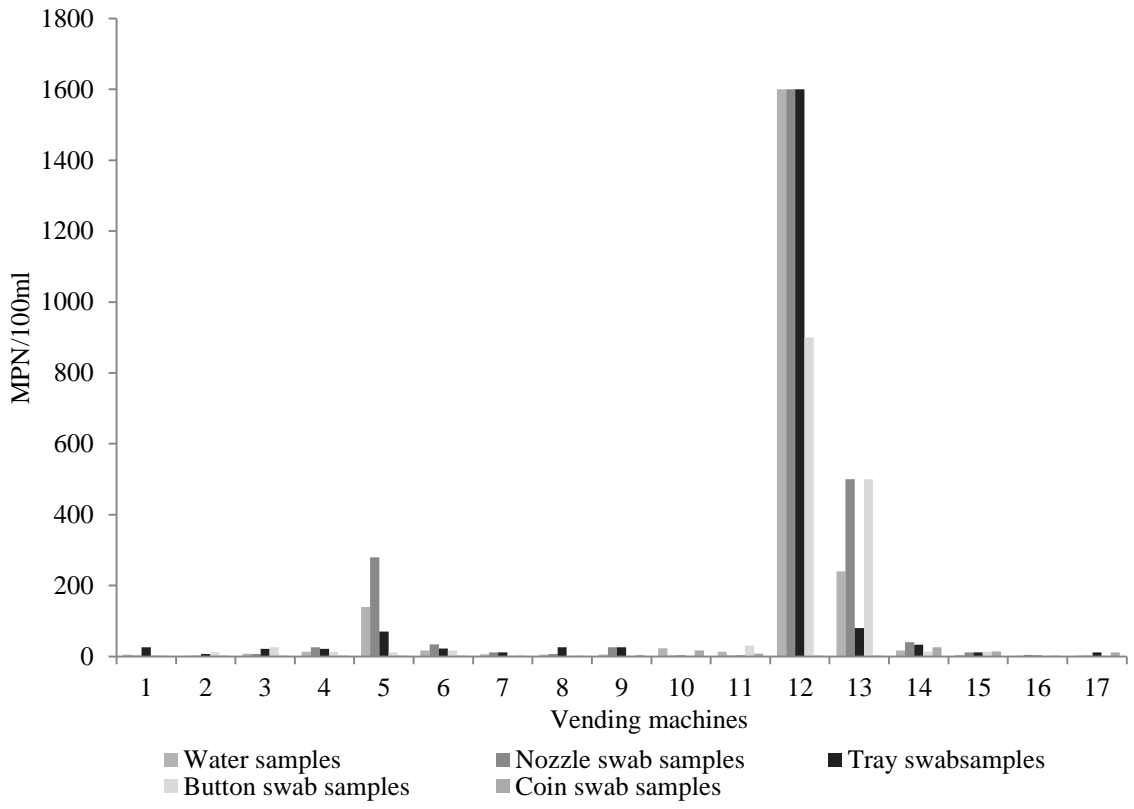
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Table 2 Morphological characteristics of isolated bacterial colony

Vending machines	Shape	Size	Colony Margin	Colony Elevation	Appearance	Optical property	Texture	Pigmentation	Gram staining
5	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Reddish pink
6	Circular	Small	Entire	Convex	Shiny	Translucent	Smooth	No	Purple
8	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Purple
12	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Reddish pink
13	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Purple
16	Circular	Small	Entire	Raised	Shiny	Translucent	Smooth	No	Purple

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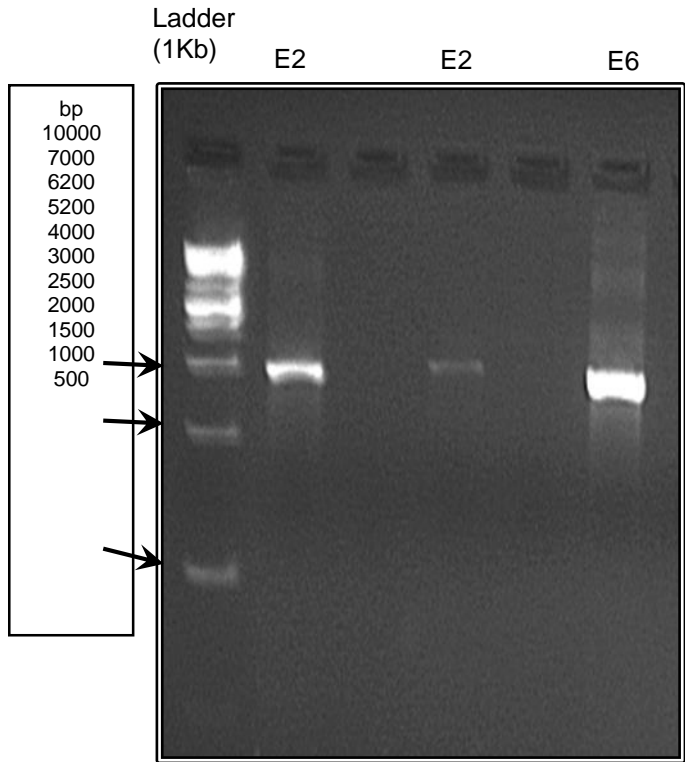


Fig. 2. Agarose gel (1%) showing amplified PCR products of 16S rDNA. Lane 1: 1Kb ladder, lanes 2 and 4: PCR product from vending machine no. 5, lane 6: PCR product from vending machine no. 12.