International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 16 No. 4 Jun. 2016, pp. 697-703

http://www.ijias.issr-journals.org/

Rapid assessment of bacteria and *Escherichia coli* in different water's sources

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ABSTRACT: A rapid protocol of detection of the bacteria in water directly by PCR has been realized without having resort to the preparation of the total DNA. The results showed that *(i*) the direct detection of the bacteria by PCR is possible under the condition to concentrate the sample of water by filtration and centrifugation; *(ii)* the sensitivity of the protocol of detection established is function of the number of bacteria in the sample; *(iii)* and it increases with the volume filtered of the sample. This protocol of detection by PCR has been used for the study of three different natural waters (source water, well water, river water), for searching the presence of *E. coli* precisely, and the bacteria in general, using primers amplifying the *uidA* gene, and those amplifying the 16S rDNA, respectively. The PCR results showed the presence of bacteria in general and *E. coli* species in river water and well water, while in the source water, no PCR amplification was obtained indicating that this water is *E. coli* free, or contaminated with a lower concentration than the detection threshold. The three samples will allow characterizing further the degree of contamination of each water. According to this work we proved also that the PT-2/PT-3 primers amplifying a fragment of the *uidA* gene (β-D-glucuronidase) may be used to reveal the presence of *E. coli* in general and *E. coli* O157:H7*,* according to the annealing temperature of the PCR.

KEYWORDS: Water, bacteria, *Escherichia coli*, PCR, *uidA* gene targeting.

1 INTRODUCTION

The water is essential for living systems, and especially for human being. It is often polluted by various germs, and problems related to water contamination are at the forefront of international concerns. The fecal pollution of water is associated with health risks, and economic losses [1]. According to the World Health Organization, third of the world population suffers from diseases caused by contaminated drinking water. Each year, 13 million people, including 2 million children die from waterborne infections. These deaths occur essentially in developing countries, but the pathogens transmitted by water occur also in developed countries, resulting in a growing danger and a significant economic problem.

The presence of enteric pathogens in water resources represents a serious risk for public health [2]. Total coliforms and *E. coli* have traditionally been considered as the best fecal indicator bacteria tracers of surface water and human fecal contamination in groundwater, respectively [3]. And according to reference [4], Enterobacteriaceae bacteria, including *Escherichia coli*, are important indicator organisms for evaluating bacteriological safety of drinking water, recreational area, or food. The Environmental Protection Agency in 1989 concerning the control of the safety of drinking water requires the specific detection and identification of *E. coli* [5].

Assessment of microbial water quality is usually performed by classical culture methods on solid medium which are simple and inexpensive. However, they have the disadvantage of being time consuming and they are not reliable [6]. These methods are unable to identify cells, termed viable but non-culturable cells (VBNC) [7], such as those bacteria stressed by certain chemicals in water [8], the UV light, pH, or the temperature change [1], [9]. These cells can become infectious [10], [11]. From a sanitary point of view, this means that the number of viable fecal bacteria is systematically underestimated by traditional culture-based methods [4]. Hence, the need for a rapid and reliable detection technique [10]. Molecular

techniques such as PCR (polymerase chain reaction) are extensively used for the rapid identification of specific pathogens, from different environment samples including water [12]. Moreover, they can be faster if combined to an easy method for PCR DNA matrix preparation. Such techniques are selective regarding the target single or mixture of gene fragments to be amplified. Furthermore, they are reliable methods, not expensive, and do not consume much time.

Highly specific to Enterobacteriaceae, 16S rDNA oligonucleotide probe allowed rapid and accurate detection of these bacteria [4]. The analysis β-(D)-glucuronidase activity targets the β-glucuronidase activity (GUR activity) [13], [14]. However, this method fails to detect the *E. coli* negative GUR. Specific PCR primer sets targeting *uidA* gene [8], [15], [16]. The primers targeting *uidA* gene have been found to be 100 % specific for the detection of *E. coli* species [6], [17].

In this work, based on the fact that variable stringency conditions during PCR should be factor to distinguish between *E. coli* O157:H7 and wild type *E. coli* and in combination with an easy protocol, to prepare biomass from the aqueous environmental sample, we propose a method to assess the presence of *E. coli* in different water samples collected from the region of Fez-Morocco.

2 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND PRIMERS

Escherichia coli strain TG1 was used for the experiments related to the comparison of the methods of the biomass concentration and those related to the determination of the PCR sensitivity threshold. The universal primers fD1 and Rs16, with the sequences, 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-TACGGCTACCTTGTTACGACTT-3', respectively [18], were used for amplification of a fragment of 1.5 kbp of the 16S rDNA of bacteria. The primers PT-2/PT-3 with the respective sequences 5'-GCGAAAACTGTGGAATTGGG-3' and 5'-TGATGCTCCATAACTTCCTG-3', were used to amplify a fragment of 250 bp from the *uidA* gene encoding β-D-glucuronidase from *E. coli* [19].

2.2 SAMPLING, CULTURE MEDIA AND BIOMASS PREPARATION

Three samples of different natural water sources (source water, well water, river water) were collected aseptically, in sterile glass vials. The samples were transported in an insulated and processed within the 6 following hours. Sample of sterile distilled water was used as negative control.

Solid LB medium (10g/l tryptone, 5 g/l yeast extract, sodium chloride 10 g/l, agar 15 g/l, pH 7.2 ± 0.2) and EMB bacterolipic (10 g/l peptone, dipotassium phosphate 2 g/l, 10 g/l lactose, 0.4 g/l eosin, methylene blue 0.065 g/l, agar 15 g/l, pH 6.8 ± 0.2) were used for enumerating bacteria, after incubation for at 37°C.

The assessment of an optimal method, for the harvesting of bacteria from the studied aqueous samples, was performed from an artificially contaminated water samples with *E. coli* TG1. 50 ml was used as starting volume. Subsequently, the water samples containing biomass was concentrated according to the following methods:

- ***i.*** Filtration through a Millipore filter with pore diameter of 0.45 µm and 13 mm in diameter. The attached bacteria on the filter were suspended, by successive vortexing up during 5 min, in 500 µl of double distilled and sterile water, followed by a final wash of the filter.
- ***ii.*** Filtration as the previously described in *(i)* but with addition of Tween 80 at 0.05% as a surfactant, to help the detachment of bacteria attached to the filter [20].
- ***iii.*** Similar to *(ii)*, but, instead of Tween 80 we used Triton X 100 at 0.5%.

The number of CFU/ml was monitored by counting on solid medium, following a serial dilution method. The performance of each method of concentration is calculated using the following formula:

$$\text{Yield of recovery} = \frac{\text{number of CFU/ml in the initial volume}}{\text{number of CFU/ml in the final volume}} \times 100$$

2.3 THE PROTOCOL USED FOR SAMPLES CONCENTRATION

A protocol of concentration for natural water sample or water artificially contaminated was developed for the direct detection of bacteria by PCR, as follows: A volume of the water sample is aseptically filtered through a Millipore membrane

with pore diameter of 0.45 µm and 13 mm of diameter. The filter is transferred to a sterile Eppendorf tube, where it is suspended in 500 µl of sterile distilled water and vortexed vigorously for 1 min. The suspension is concentrated by centrifugation at 12 000 g for 15 min at 4˚C. The pellet was suspended in a volume of 50 ml of sterile water. 2 µl were taken and added to the PCR reaction mixture.

The determination of sensitivity threshold of protocol of detection by PCR was run out, using decreasing biomass load in the test sample ranging from 0 CFU/ml to 2600 CFU/ml. According to the concentration method selected among the three compared methods, the obtained concentrate was used directly for PCR analyses. First, the presence of bacteria was assessed by PCR, with primers fD1/Rs16, using the PCR mixture, and the following cycling steps program, respectively. 2 µl from the final concentrate were added for a final volume of 20 µl containing: 1 µM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1U of enzyme Taq polymerase (Promega), 2 µl of enzyme buffer. The PCR was carried out with an initial denaturation at 95°C (2 minutes), followed by 35 cycles containing each one: denaturation at 94°C for 30 s, annealing 55°C for 40 s and elongation at 72°C for 1min 30 s. Followed by a final cycle at 72°C for 10 min. PCR with PT-2/PT-3 primers targeting the *uidA* gene encoding β-D-glucuronidase were used to detect the presence of *E. coli* [19]. The same mixture was used for PCR, as described previously, with the following cycling steps: an initial denaturation at 95°C (2 minutes), followed by 35 cycles containing each one, denaturation at 94°C for 30 s, annealing 55°C for 40 s and elongation at 72°C for 30 s. Followed by a final elongation step at 72°C for 10 min. According to reference [21] the PCR products were visualized under UV light after a 1% agarose gel electrophoresis. The gel was stained by ethidium bromide solution 0.5 mg/ml.

3 RESULTS

3.1 EVALUATION OF THE BIOMASS CONCENTRATION METHOD

The analyses of a water sample require a step for a biomass preparation of the studied water. Filtration techniques are methods of choice. The water's samples were filtered through a Millipore filter with pore diameter of 0.45 µm and 13 mm in diameter. The detachment of the attached biomass, into a determined small volume of sterile water, was performed under vigorous vortexing in the presence or the absence of a tension-active compound, such as Tween 80 or Triton X100. The results of the comparison of the three methods of concentration are shown in Fig. 1.

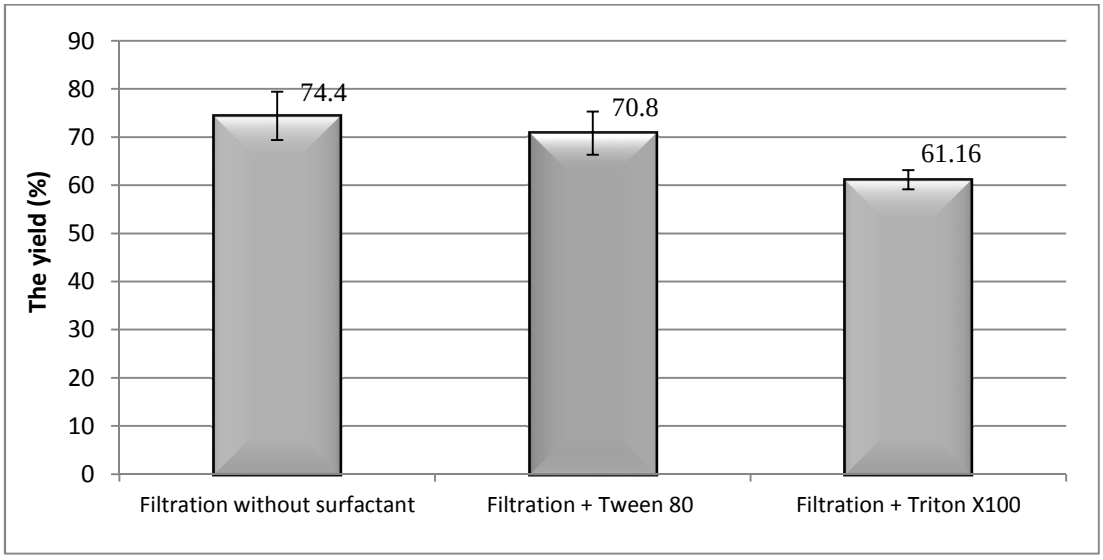

Fig. 1. The yield of recovery for the three methods of concentration of suspension of E. coli.

3.2 ASSESSMENT OF THE ARTIFICIALLY CONTAMINATED SAMPLES

First experiments performed directly from the studied samples, with the primers (e.g. fD1/ Rs16 and PT-2/PT-3), showed no amplification of the targeted DNA fragments (Fig. 2). In Fig. 2, it is shown that the decrease of the initial biomass load in the PCR mixture, is accompanied by a reduction of the PCR products.

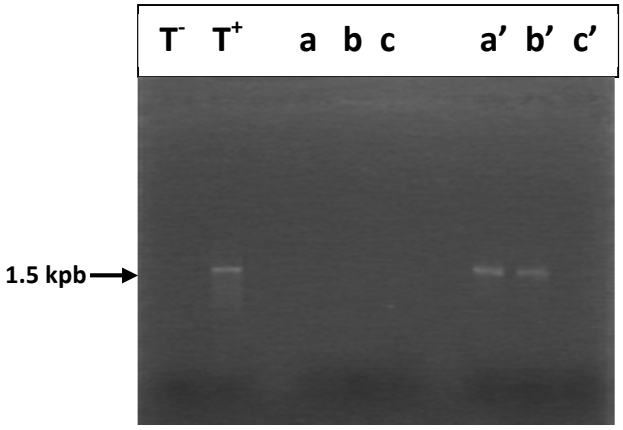

Fig. 2 Agarose gel (1%) electrophoresis analysis of PCR products, from the three artificially contaminated samples (no concentrated a, b, c, and concentrated a', b', c'), using the primers fD1/Rs16. T⁻: negative control (concentration of a sterile water sample); T⁺: positive control (PCR from E. coli TG1 genomic DNA); a: sample containing 2600 CFU/ml; b: sample containing 180 CFU/ml; c: sample containing 50 CFU/ml; a': concentration of a sample containing 2600 CFU/ml; b': concentration of a sample containing 180 CFU/ml; c': concentration of a sample containing 50 CFU/ml.

3.3 ASSESSMENT OF NATURAL WATER SAMPLES

100 ml and 1 liter of sample were concentrated, and then the PCR was performed directly from the biomass concentrate (without DNA purification, neither DNA extraction). The results of the detection of bacteria by primers FD1 / RS16 (Fig. 3 (A)), obviously show that the samples analyzed contain bacteria with different loads.

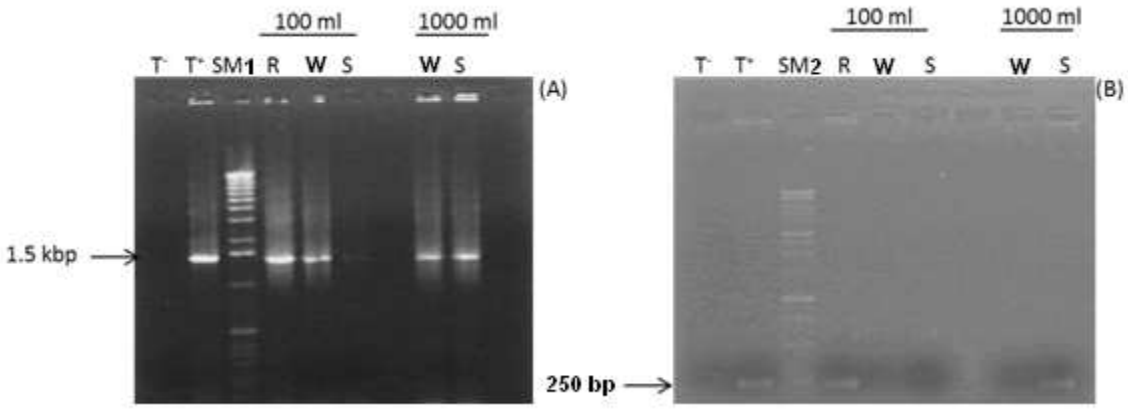

Fig. 3. Agarose gel (1%) electrophoresis analysis of products PCR, after concentration of 100 ml and 1liter, using the primers fD1/ Rs16 (A) and using the primers PT-2/PT-3 (B). SM1: DNA ladder as a size standard; SM2: DNA ladder (1kb plus; Promega); T⁺: positive control (E. coli strain TG1); T⁻: negative control (1L of the sterile distilled water dealt with the same protocol); R: the sample of the river water; W: the sample of the well water; S: the sample of source water.

Along with PCR analysis, the same germs sought by PCR, were monitored and enumerated by membrane filtration method (Table 1).

Table 1. Comparison between the results of the PCR and the membrane filter technique

	Samples	PCR detection		Number of CFU/100 ml of samples
		100 ml	1000 ml	
Total bacteria	The river water	+	Not used	20.5 10^3
	The well water	+	+	18 10^3
	The source water	+/-	+	3.6 10^3
E. coli	The river water	+	Not used	10 10^3
	The well water	-	+	4.2 10^3
	The source water	-	-	8

4 DISCUSSION

Concerning the comparison of the three methods of concentration used (Fig. 1), the ANOVA study showed that there is no significant difference (at $p < 0.05$) between the three methods of biomass harvesting compared. These results are similar to those found by [22], who have shown that the use of Tween 80 as a surfactant causes a non-significant change in the yield of harvested bacteria compared to the control. We used the method "*filtration without surfactant*" for the protocol that we established.

Then, we used the established protocol for detecting the presence of bacteria in samples contaminated artificially with different bacterial loads. Results (Fig. 2) showed that the PCR performed from these samples without concentration is always negative regardless of the microbial load. While the detection of these bacteria becomes possible for samples containing 2600 CFU/ml and 180 CFU/mL, after their concentration by the established protocol. However this protocol does not allow the detection of the presence of bacteria with a load of 50 CFU/ml.

Natural water samples are assessed by the established protocol. The agarose gel (1%) electrophoresis analysis of products PCR (Fig. 3) show that the river water showed the intense signal of PCR products, followed by well water, and source water, respectively. Among these bacterial loads from the analyzed samples, the presence of *E. coli* species has been detected using the primers set PT-2/PT-3. This primer's set has been intended to amplify the *uidA* gene of *E. coli* O157: H7 [20]. It has two double-mismatches from the wild-type gene *uidA*. The gene *uidA* of *E. coli* O157:H7 possess a guanine residue (rather than the thymine residue, found in wild-type *E. coli* in the 3' end, at position 92) [23]. Though, the change in the stringency conditions of the PCR reaction, mainly the change of the annealing temperature will affect the specificity of the used primers toward *Escherechia coli* O157:H7 to amplify *uidA* from other *E. coli*. PCR conditions used in the analyses by [20], mainly the annealing temperature of 64°C allowing higher specificity for *uidA* gene O157: H7. However, the same primers (at an annealing temperature of 55°C) are demonstrated to target *E. coli* TG1, a non-pathogenic species as shown in Fig. 3 (B). Thus, suggesting the use of primers PT-2/PT-3 as marker for both, *E. coli* species and the pathogenic *E. coli* O157: H7, ultimately under specific PCR cyclization programs. The PCR results represented in Fig. 3 (B) show that the sample of the river is contaminated with *E. coli*, and containing at least a total greater than or equal to 9.10^3 CFU per volume of filtered water (100 ml), which is the detection limit of this protocol using PCR. This is a rough estimate of the contamination. Because PCR is a qualitative and not quantitative technique and provides information on the presence or absence of bacteria in the sample sought. For analysis of 1 liter, the PCR reaction is positive for the sample from the well water, which means that it contains at least 9.10^3 CFU per volume of filtered water (1 liter). While no amplification was detected for the source water sample, suggesting that this sample would be free *E. coli*, or containing a total number of *E. coli* well below 9.10^3 CFU per volume of filtered water (1 liter).

Along together, the results of PCR and those from the agar media (Table 1), show that:

- The primer's set PT-2/PT-3 might be used to detect total *E. coli*, and the pathogenic *E. coli* O157:H7 according to the annealing temperature used during the PCR.
- The sensitivity of detection by PCR according to the established protocol, improves with the increase of the volume of the sample filtered.
- The enumeration of bacteria by membrane filtration confirms the presence of bacteria in the three samples as was shown by the PCR results.
- The enumeration of bacteria by membrane filtration technique confirms the presence of *E. coli* in river water, and that of the well.

- The membrane filtration technique shows that the number of *E. coli* in the source's water is lower to detection doorstep of the established protocol, which explains why this protocol did not detect it. Confirming so, the sensitivity of this protocol.

5 CONCLUSION

The direct detection of bacteria of the genus *Escherichia* in water by PCR is a reliable tool for the assessment of the degree of contamination of the analyzed water. DNA matrix preparation is occurred by providing bacteria directly to the PCR mixture. This protocol allowed us to detect the presence of bacteria by targeting the 16S rDNA. Similarly we detected the presence of *E. coli* by amplification of a fragment of the *uidA* gene by using the primer's set PT-2/PT-3, which suggests their usefulness to detect both, total *E. coli* and *E. coli* O157:H7, only by controlling the cyclization conditions of PCR, mainly the annealing temperature. The sensitivity of detection by PCR according to the protocol established improves when we increase the volume of the filtered sample. The results also show that sterile water sample processed by the same protocol, do not have amplification of the expected fragment, in contrast to the results from *E. coli* TG1. That implied the reliability of our protocol, which does not use the DNA extraction of total bacteria in the sample.

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