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Detection of *RpoS* Gene in *Escherichia Coli* O157:H7 and non-O157 and Their Survival Pattern in Water Treatment Methods

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Abstract: This study aimed to detect presence of *RpoS* gene in *Escherichia coli* O157:H7 and non-O157 and investigate their survival pattern in different water treatment methods. A total of fifteen serologically and molecularly identified *E. coli* was selected from a previous work, out of which eight were *Escherichia coli* O157 and seven were *E. coli* non-O157. From among these, S30 and S89 identified isolates served as presentative *E. coli* O157:H7 and non-O157 respectively for survival studies. The water treatment methods used employed included: use of silver, lime, storage, acidification (low pH), high temperature and *Moringa oleifera.* Survival pattern of the test organisms under the influence of these methods were carried out using standard techniques. Molecular detection of stress response gene, *RpoS*, in the fifteen (15) test organisms was performed following manufacturer's instruction. Results showed that for both test organisms, silver was bactericidal at high concentration while storage allows their survival up till 21 days though with a reduction in cfu. Both organisms showed low survival at pH 9 while *E. coli* O157:H7 and non-O157 could survive at pH 4 and 6 respectively. *E. coli* O157:H7 survived better than non-O157 at high concentration of lime. While both survived at low temperature, *E. coli* O157:H7 survive better at 60^{o}C. Sunlight and chlorine showed mild and complete bactericidal action respectively with increased exposure time for both test organisms. Moringa oleifera was only effective at a reduced concentration on the two organisms. Detection of *RpoS* genes showed that only 66.7% carried the gene in them while 33.3% did not. Findings from this study show that the possession of stressed genes in bacteria causing waterborne disease could allow these organisms to survive water treatment methods adopted in many under developed countries or rural communities. This suggests a threat to health of these communities.

Keywords: *E. coli O157: H7; E. coli non-O157; water treatment methods; RpoS genes; Survival pattern.*

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Introduction

The availability of potable water is a function of appropriate treatment methods applied to it. Several treatment methods which include biological, physical and chemical methods are being employed to ensure availability of potable water to communities or homes. Consequently, reducing incidences of waterborne diseases in communities or households. However, in under developed countries or rural communities where potable water is not available to the residents, several readily available and cheap methods of water treatment are employed to safeguard their health. Among these methods include use of silver, lime, acidification (low pH), storage, sunlight (Solar Disinfection), low and high temperature and *Moringa oleifera* seed. As a matter of fact, some of these methods may not actually be effective in reducing bacterial load of the water as some enteric bacteria causing waterborne diseases have defied these treatments methods.

Among these enteric bacteria is *Escherichia coli* which exhibits a high degree of catabolic flexibility, which conferred a clear fitness (survival) advantage in its secondary habitats such as soil and water (Ihssen and Egli, 2005). Survival of *E. coli* in water varies depending upon species, strain, and environmental pressures, but the mechanisms governing their fate are poorly understood (Gordon *et al.*, 2006; Semenov *et al.*, 2009). *Escherichia coli* O157:H7 poses health risk when they are excreted into the environments. Various studies have documented the ability of *E. coli* O157 to survive for extended period of time within secondary habitats including water, sediment, and vegetation (LeJeune *et al.*, 2004; LeJeune *et al.*, 2004; Islam *et al.*, 2004; Semenov *et al.*, 2009; Oliveira *et al.*, 2012; Saxena and Sewak, 2016).

Escherichia coli O157 may survive and even grow in sterile freshwater at low carbon concentrations, which is in contrast to the common conception that the organism will die out over time in such strongly carbon-limited environments (Vital *et al.*, 2008). Moreover, because of the production of *RpoS*, *E. coli* is able to rapidly adapt to and tolerate diverse stress conditions. It was thus revealed that high osmolarity, extremes and fluctuations of temperature, low pH and low growth rate induce *rpoS* in *E. coli* cells (Elsas *et al.*, 2011). Also, *E. coli* can enter a dormant, that is, viable but non-culturable state in which the cells cannot be easily recovered on standard laboratory media, but are still present as viable cells. This state can be triggered by stress conditions that are imposed, for instance, by low temperature (for example, 4 °C) or toxic metals for example, copper, lead, mercury and cadmium (Bhagat *et al.*, 2016). Although, the resistance to starvation of *E. coli* leads to its persistence in the environments, predation, substrate competition and antagonisms by indigenous microflora lead to a negative effect on its survival in the environment (Wcislo and Chrost, 2002; Elsas *et al.*, 2011).

Several reports on outbreak of waterborne diseases caused by *E. coli* O157 and non-O157 have been documented but there have been little or no information on their survival in the environment and under the influence of water treatment methods. And studies of factors affecting the survival of *Escherichia coli* in natural waters would be of great interest due to the importance of this microorganism as the primary index of faecal contamination of water (Edberg *et al.,* 2000; WHO, 2006). Therefore, this study aimed to detect the presence of *RpoS* gene in *Escherichia coli* O157:H7 and non-O157 and investigate their survival pattern in different readily available and cheap water treatment methods.

Materials and Methods

Test organisms: From a previous study, a total of fifteen (15) biochemically, serologically and molecularly identified *E. coli* were selected. The identified isolates were S2, S10, S30, S35, S70, S71, S89 and S152 from well, S3B, S82 and S177 from stream, S31 and S33 from borehole and S34 and S202 from pipe-borne water samples. Among which, eight (8) were identified as *E. coli* O157:H7 and seven (7) were *E. coli* non-O157. Isolate S30 and S89 were used as presentative *E. coli* O157:H7 and *E. coli* non-O157 respectively for the survival studies while the whole fifteen identified isolates were used for molecular studies.

Experiments: Investigation on physicochemical characteristics of *E. coli* O157 (S30) and *E. coli* non-O157 (S89) survival in water were performed under varied physicochemical factors using the standard techniques employed by Wcislo and Chrost (2000) and Sakyi and Asare (2012). These factors included low and high temperature, pH, chlorine, silver nitrate, lime, sunlight and *Moringa oleifera.*

Preparation of Inoculum for Survival Experiments: The inoculum was prepared as described by Wcislo and Chrost (2000) and Al-Qadiri *et al.* (2011). S30 and S89 were grown in 50 ml of Tryptone Soy Broth (TSB) (Lab M, LAB011 U.K.) and incubated at 37°C for 24 hours. After incubation, 10 ml of S30 and S89 broth were centrifuged using bench top centrifuge (AccuSpin model 400 bench top centrifuge, Fisher Thermo Scientific, Pittsburgh, PA) at 3000 rpm for 15 minutes. To eliminate any effect of broth components and bacterial metabolites, the resultant pellets were re-suspended in 10 ml of sterile 0.85% (wt/vol) saline solution and centrifuged as before. After the second centrifugation, the supernatant was decanted, and the pellets were re-suspended in 10 ml of the same sterile saline solution corresponding to approximately 10^6 cfu/ml.

Effect of Silver Nitrate: Different concentration of 0.01mg/l $AgNo_3$ was prepared by doubling-fold dilution method to give 1.0, 0.5, 0.25, 0.125 and 0.0625 concentrations in the test tubes. After which, one ml of dilution 10^6 of the prepared inoculums was added to each of the test-tubes. The control test-tube did not contain silver nitrate. The time of exposure to $AgNO_3$ was an hour.

Effect of Storage: nine test-tubes consisting of sterile 9ml water samples each inoculated with 1ml of dilution 10^6 of the prepared inoculums were stored at room temperature for 24days. The presence and proportion of test organisms at every 3 days interval were investigated and recorded. The test-tubes serving as control were plated immediately to detect the presence and proportion of *E. coli* O157 and non-O157.

Survival at Low and High pH: Nine ml water sample each was dispensed into seven test-tubes and the water in each test-tube was adjusted to the desired pH (3.0, 4.0, 5.0, 6.0, 7.0 and 9.0) with 1M phosphate buffer. The tubes were sterilized for 15 mins at 121°C. The pH of the water did not change after autoclaving. The tubes were inoculated with 1 ml of dilution 10^6 of the prepared inoculums. Also, there was a control with no adjusted pH which was inoculated with the same volume of prepared inoculums. The test-tubes were incubated for an hour.

Effect of Lime: The limes were washed cleaned after which was wiped with ethanol and then peeled aseptically in order to extract the liquid content. It was filtered using No 1. Whiteman filter paper. Different concentrations of the filtrate were prepared by doubling-fold dilution method to give 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml concentrations in different test tubes. Then 1 ml of dilution 10^6 of the prepared inoculums was added to each of the test-tubes. The control test-tube did not contain lime. The time of exposure to lime was an hour.

Survival at Low Temperatures: Sterile 9 ml water sample in different sterile test-tubes were inoculated with 1ml of dilution 10^6 of the prepared inoculums. Then they were aseptically stored at 22 ^oC, 8 ^oC, and 0^oC for 5, 7, and 2 days, respectively. These storage intervals were selected because some individuals keep drinking water at room temperature for 2 to 5 days, whereas others prefer to refrigerate water and consume it within a week. The storage at 22^oC served as control.

Effect of High Temperature (40 ^oC, 50 ^oC and 60 ^oC)**:** Sterile 9 ml water sample in different sterile test-tubes were inoculated with 1ml of dilution 10^6 of the prepared inoculums. The test-tubes were heated till the contents were brought to 40 ^oC, 50 ^oC and 60 ^oC for about 5mins. The test-tubes serving as control was not exposed to heating.

Effect of Sunlight: This was carried out on an intense sunny day. Sterile 9 ml water sample in different sterile test-tubes were inoculated with 1ml of dilution 10^6 of the prepared inoculums. The test-tubes were exposed to intense sunrays at different time interval with the exception of the control.

Effect of Chlorine: Sterile 9 ml water sample in different sterile test-tubes were inoculated with 1ml of dilution 10^6 of the prepared inoculums. 0.1 ml of 0.25mg/ml of chlorine was added to each test-tube except the control. The exposure time was 30secs., 60secs., 90secs. and 120secs.

Effect of Aqueous Extract of *Moringa oleifera* Seeds: Seeds of *M. oleifera* were collected from *M. oleifera* tree. The seeds were de-shelled to get the kernels. The Seed kernels were further dried at ambient temperature for five days. The white kernels were milled into a fine powder using a blender (Marlex Electroline Dabhel, Daman). Then 10g was weighed and soaked in sterile 100ml of distilled water in a beaker for 24hrs. The extract was then filtered using a filter paper and different concentrations, 0.05, 0.1, 0.15, 0.20 mg/ml and neat of the extract were prepared. Then 9 ml sterile water sample in a conical flask inoculated with 1 ml of dilution 10^6 of the prepared inoculums was used for the experiment. From each concentration of the extract, 1ml was taken aseptically and poured into the content of the conical flask and mixed thoroughly. This was done because it has been documented that, 4α-4-rhamnousyloxy-benzyf-isothiocynate, the active antimicrobial agent in *M. oleifera* is readily soluble in water and is non-volatile (Bichi, 2013). The mixture was allowed to stand overnight at room temperature. The negative control conical flask did not contain any *M. oleifera*. One ml from each conical flask was taken and plated on EMB agar plate and incubated at 24hrs at 37^oC.

Recovery of *E. coli* O157 and non-O157 on EMB Agar. S30 and S89 counts and identification were determined by plating 1 ml from each test-tube of the test experiment on EMB Agar after the exposure duration of each test experiment. These were incubated at 37°C for 24 hrs. Thereafter, the number of *E. coli* O157 (S30) and non-O157 (S89) were counted and recorded in Log_{10} cfu/ml

Molecular Detection of RpoS Genes

E. coli O157 (n=8) and E. coli non-O157 (n=7) were grown overnight at 37oC and standard PCR techniques were used, following manufacturer's instruction. The primer used is shown in Table 1.

Table (1): PCR primers used in this study

Primer set	Target gene	Sequence (5'→ 3')	Size of PCR Product (bp)	References
A	*E. coli* O157 *RpoS*	5'GCGTTGCTGGACCTTATC3' 3'GAATAGTACGGTTTGGTTCATAAT5'	250	Parry-Hanson *et al.* (2010)

Results

The results from this study showed a characteristic difference in the survival of *E. coli* O157:H7 and *E. coli* non- O157 under varied physicochemical factors as follows:

Effect of silver nitrate: Results showed that the two strains exhibited the same response to silver nitrate. At higher concentrations of dilution 1, 0.5, 0.25, 0.125, silver nitrate was bactericidal on the two strains while few colonies were able to survive at lower concentration of dilution 0.0625, 0.0312, 0.0156 (Fig. 1).

Effect of storage: It was observed that both strains persisted in drinking water up till 21days at room temperature. Although, their $\log_{10}$ cfu/ml decreased with days when compared with the control, that is, from 7.64 $\log_{10}$cfu/ml to 6.42 $\log_{10}$ cfu/ml and 7.68 $\log_{10}$ cfu/ml to 6.95 $\log_{10}$cfu/ml for *E. coli* O157 and non-O157 respectively (Fig. 2).

Effect of pH: The result in Fig. 3 shows that while *E. coli* O157 was able to survive at pH 4, *E. coli* non-O157 was unable to survive at that same pH. Although, it was observed that *E. coli* non-O157 was able to survive at slightly acidic condition (pH6). It was also shown that the survival of both strains was low at pH 9 with reduced $\log_{10}$ cfu/ml.

Effect of lime: Fig 4 depicts that *E. coli* O157 survived better at higher concentration (1.0 and 0.5) of lime than *E. coli* non-O157. However, their $\log_{10}$ cfu/ml increased inversely with concentration of the lime.

Effect of low and high temperature: Varied survival pattern of the two strains were observed under low and high temperature (Fig. 5). Both strains survived at low temperature of 4°C to 20°C but as the temperature increased, there was a better survival of *E. coli* O157 than *E. coli* non-O157.

Effect of sunlight: Sunlight experiment revealed mild bactericidal effect of sunlight on the two strains as their $\log_{10}$ cfu/ml decreased with length of exposure from 6.87 $\log_{10}$ cfu/ml to 5.0 $\log_{10}$cfu/ml and 7.60 $\log_{10}$cfu/ml to 6.30 cfu/ml of *E. coli* O157 and non-O157 respectively (Fig. 6).

Effect of Chlorine: Chlorine exhibited a quick and total bactericidal effect on both strains as there was no survival even after 30sec of exposure (Table 2).

Effect of aqueous extract of *Moringa oleifera*: Fig. 7 shows that the extract was more effective in reducing the $\log_{10}$cfu/ml of the two strains at higher concentration than at lower concentration. However, it was observed that at low concentration of dilution 0.062, there was an increase in $\log_{10}$cfu/ml in both strains when compared with the control. This revealed that the extract was utilized as nutrient by

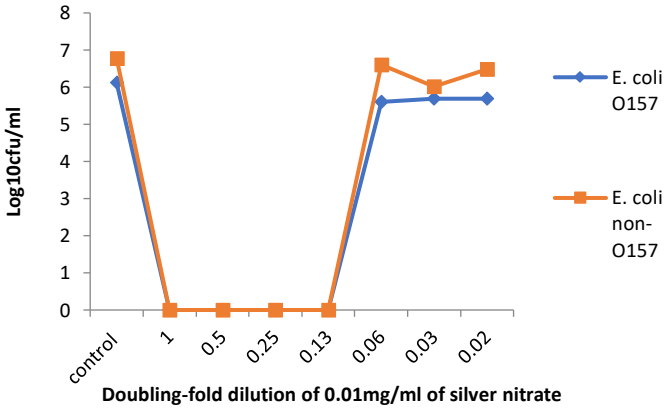

Fig (1): Effect of Silver Nitrate on E. coli O157 and E. coli non-O157 in Drinking Water

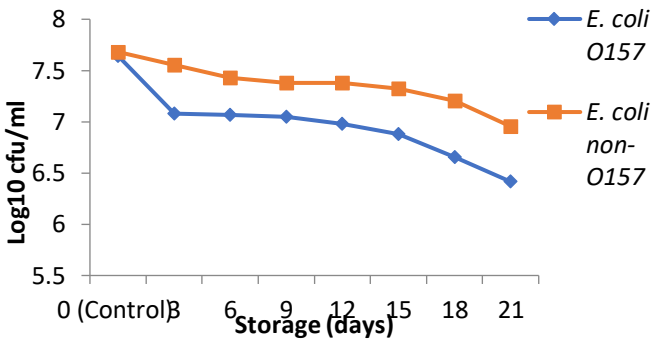

Fig (2): Effect of Storage on *E. coli* O157 and *E. coli* non-O157 in Drinking Water

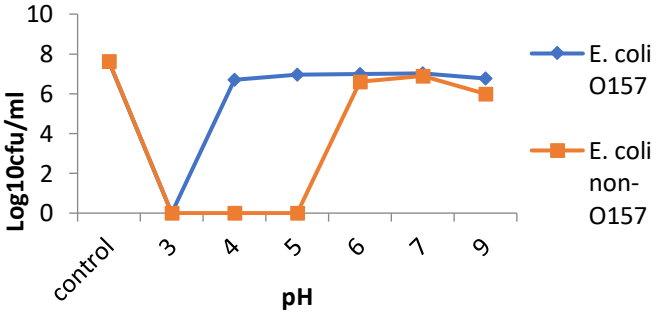

Fig (3): Effect of pH on *E. coli* O157 and *E. coli* non-O157 in Drinking Water

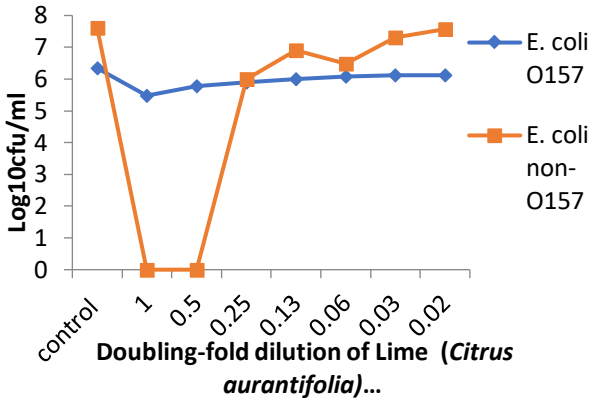

Fig (4): Effect of lime on *E. coli* O157 and *E. coli* non-O157 in Drinking Water

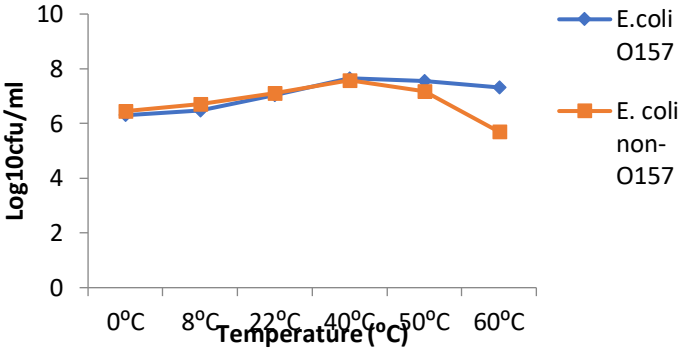

Fig (5): Effect of Low and High Temperature on *E. coli* O157 and *E. coli* non-O157 in Drinking Water

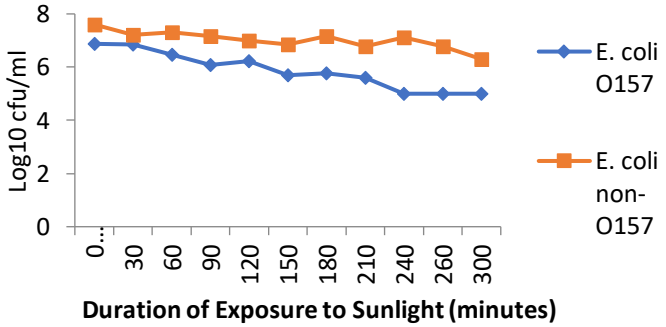

Fig (6): Effect of Sunlight on *E. coli* O157 and *E. coli* non-O157 in Drinking Water

Table (2): Effect of 0.25mg/ml of Chlorine on *E. coli* O157 and *E. coli* non-O157 in Drinking Water

Exposure (secs.)	Time	*E. coli* O157 (Log $_{10}$cfu/ml)	*E. coli* Non-O157 (Log$_{10}$ cfu/ml)
30		0.00	0.00
60		0.00	0.00
90		0.00	0.00
120		0.00	0.00
Control		6.1139	7.6335

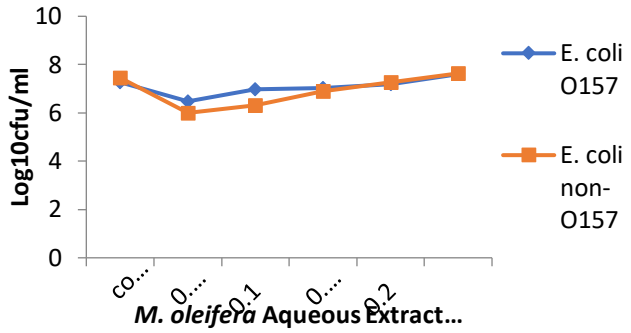

Fig (7): Effect of *Moringa oleifera* Aqueous Extract on *E. coli* O157 and *E. coli* non-O157 in Drinking Water

Molecular detection of *RpoS* gene: figure 8 revealed that some of the isolates possess stress response gene (*RpoS*) which allowed their survival in a secondary habitat (water). All the isolates were positive for *RpoS* gene of 250bp except for isolates in lane 4, 5, 6, 8 and 9 which showed absence of the gene in them, that is, only 66.7% carried the gene in them while 33.3% did not.

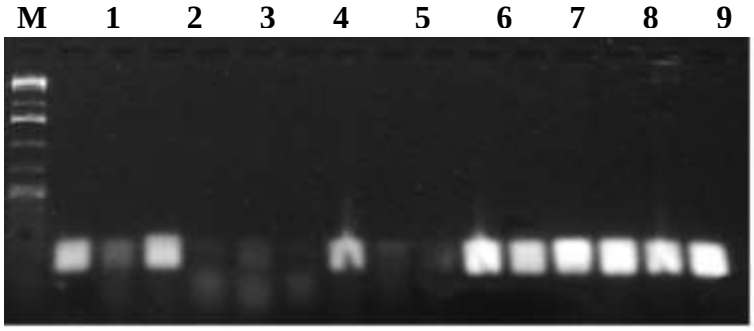

Fig (8): Gel Electrophoresis for *RpoS* gene (250p)
Lane 1-8 (*E. coli* non-O157), Lane 9-15 (*E. coli* O157), Lane M (100bp DNA size marker) Lane 1 (S71 from well sample), Lane 2 ((S177 from stream sample), Lane 3 (S31 from borehole sample), Lane 4 (S70 from well sample), Lane 5 (S89 from well sample), Lane 6 (S152 from well sample), Lane 7 (S202) from pipe-borne water sample), Lane 8 (S34 from pipe-borne sample), Lane 9 (S33 from borehole sample), Lane 10(S3B from stream sample), Lane 11 (S2 from well sample), Lane 12 (S35 from well sample), Lane 13 (S82R from stream sample), Lane 14 (S30 from well sample) and Lane 15 (S10 from well sample).

Discussion

In various homes and industries where water is being consumed or used, several water purification methods are being adopted to eliminate or reduce the microbial load of the water. But surprisingly, few pathogenic microorganisms have devised several mechanisms in them to survive these purification methods. Among these treatment methods, silver has been used as an effective water disinfectant for many

decades (Kim *et al.*, 2004). Both the Environmental Protection Agency (EPA) and the World Health Organization (WHO) regard silver as safe for human consumption (WHO 1996). Gram-negative bacteria appear to be more sensitive than Gram positive species because gram-positive species have a thicker peptidoglycan layer than do gram-positive species (Kawahara et al. 2000). Its disinfection was found to be toxic to microorganisms and this was confirmed in this study as it inhibited the growth of the two strains of *E. coli* after an hour exposure. This corroborates the findings of Russell and Hugo (1994) who reported that silver was found effective at preventing growth of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* in water supplies. Application of little quantity of silver is not health threatening, but in larger amounts, some silver compounds may be toxic, because silver ions have a high affinity for sulphur hydryl and amino groups, and therefore complexation with amino acids, nucleic acids and other compounds may occur in the body (Silvestry-Rodriguez et al., 2007).

Effect of storage in this study revealed that the organisms were able to persist in sterile drinking water up till 21 days, although, with a reduction in their $\log_{10}$cfu/ml at the end of 21 days. This corroborates with the study of Berthe *et al.* (2013) who also recorded that persistent strain of *E. coli* were able to survive up to 14 days. This is further validated by the observation of Adesioye and Ogunjobi (2013) who reported that *E. coli* survive better in sterile drinking than in unsterile drinking water and that a general decrease of *E. coli* count with time was noticed.

Also, it was observed that *E. coli* O157:H7 survived better than *E. coli* non-O157 at low pH. This finding strengthened the studies by other researchers who reported the ability of *E. coli* O157 to survive in an acidic medium (Oshoma *et al.*, 2009; Albashan, 2009; Parry-Hanson *et al.*, 2010). Although, *E. coli* O157 strains still showed different capacities to survive in acidic environment, they are superior in their survival over *E. coli* non-O157 (Bergholz and Whittam, 2007). Variation in *rpoS* induction levels might explain the variability in acid resistance of different *E. coli* O157:H7 strains. The effectiveness of lime (*Citrus aurantifolia*) in water purification and in controlling pathogens causing intestinal disorder has been documented (Nannapaneni *et al.,* 2008; Jacob and Sumathy, 2010; Ojiezeh *et al.*, 2011). Although, this study showed that *E. coli* O157:H7 was still able to survive at high concentration of the lime possibly due to its ability to adapt to an acidic environment, *E. coli* non O157 was not. This is of health significance in a community setting where lime is being used in purifying water. This suggests that *E. coli* O157 will still survive in such water. Researchers have also revealed that the use of lime in combination with sunlight will solve this problem (http://releases.jhu.edu).

For solar disinfection (SODIS) which uses the sun's energy to provide an economically feasible means of providing safe drinking water. SODIS is a free and effective method usually applied at the household level and is recommended by World Health Organization as a viable method for household water treatment and safe storage. Under the right conditions (intense sunlight), solar water disinfection, or SODIS, could solve the problem of water contamination. Studies have shown that when unpotable water sample stored in transparent bottle is exposed to sunlight for about five or six hours or 48 hours in a cloudy weather, there was a significant reduction in coliform forming unit of *E. coli* after the exposure (Wcislo and Chrost, 2000; Esemikose and Azeez, 2013). These confirm the findings on sunlight effect in this study. The ultraviolet radiation from sunlight creates highly reactive oxygen species such as superoxide (O^{2-}), hydrogen peroxides (H_2O_2), and hydroxyl radicals (OH^-) which in turn oxidize microbial cellular components such as nucleic acids, enzymes, and membrane lipids, which kill the microorganisms (Esemikose and Azeez, 2013). Effect of low and high temperature on the two strains showed that the two strains were able to survive at low temperature though, their count were low compared to their control. However, *E. coli* O157:H7 survived better when exposed to high temperature than *E. coli* non-O157. This may probably be as a result of the induction of the sigma factor, *rpoS,* which allows *E. coli* O157 to overcome environmental stresses. In a similar study, Ansay *et al.* (1999) reported that *E. coli* O157:H7 survives freezing with some decline in cfu. Equally, Clavero and Beuchat (1996), recovered 5 strains of *E. coli* O157:H7 at 60°C for 0, 15 and 30 minutes. This signifies that, *E. coli* O157:H7 could survive temperatures above 45°C. *Moringa oleifera* aqueous extract was able to reduce count of both strains in drinking water samples. This is made possible due to an active antimicrobial agent, 4α-*4-rhamnousyloxy-benzyf-isothiocynate,* present in the seed and it is readily soluble in water. This finding is validated by observations made by some researchers (Suarez *et al.*, 2003; Suarez *et al.*, 2005; Fisch *et al.*, 2004; Thilza *et al.,* 2010; Bukar *et al.*, 2010 and Bichi, 2013) who reported the ability of this plant extract in purifying drinking water. Thus, reducing the microbial load of the water sample.

The molecular screening for the presence of *RpoS* gene in the selected isolates showed that majority of *E. coli* O157:H7 carried this stress genes in them which may probably suggests their ability to withstand environmental stress. This is in concordance with the study by Parry-Hanson *et al.* (2010) who reported *RpoS* gene expression in acid-adapted *Escherichia coli* O157:H7 during lactoperoxidase and lactic acid challenge in tryptone soy broth. *RpoS* is the primary sigma factor for global regulation of genes associated with environmental stresses (Nickerson *et al.,* 2004). Bacterial cells

exposed to a large variety of harmful agents respond by increasing the cellular concentration of a stress sigma factor which replaces the house keeping sigma factor on the RNA polymerase enzyme, thereby changing its regulatory properties (Nickerson *et al.,* 2004).

In conclusion, this study has revealed the effectiveness of some water treatment methods as these methods do not support the survival and growth of *E. coli* O157:H7 and non-O157. However, some were found to be ineffective as these strains of *E. coli* could still survive after exposure to them. Moreover, the study revealed that stress sigma factor (*RpoS* gene) assists bacteria in surviving harsh conditions (treatment methods). In addition, bacteria causing waterborne diseases will survive if adequate concentration and exposure to agents used in water treatment methods are not observed. Consequently, in communities or households where this invaluable information, on survival pattern of bacteria causing waterborne disease, is lacking, high incidence of waterborne diseases will often be reported.

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