PCR CHARACTERIZATION OF *ESCHERICHIA COLI*

[adapted, in part, from Omar and Barnard (2014) Detection of diarrhoeagenic *Escherichia coli* in clinical and environmental water sources in South Africa using single-step 11-gene m-PCR. *World Journal of Microbiology and Biotechnology* 30: 2663–2671; www.ncbi.nlm.nih.gov/pmc/articles/PMC4150989]

Principle and Purpose

This laboratory exercise is intended to provide students the opportunity to apply molecular-based techniques to identify environmental isolates of *Escherichia coli* that possess genes encoding for toxins relevant to virulence. This exercise builds upon the prior experiment, *Membrane Filter Detection of Coliforms* and *Enterococci*. In this regard, this particular exercise is presented as if students were successful in isolating an environmental *E. coli* strain.

Note: If students were not able to isolate an environmental strain of *E. coli*, one shall be provided by the laboratory instructor.

Moreover, if an isolate has been recovered and as previously emphasized, it is important to confirm the identity of the environmental isolate as *E. coli* by using the IMViC series of biochemical tests, EMB agar, and EC broth as described in the prior laboratory exercise (*Membrane Filter Detection of Coliforms and Enterococci*).

In this exercise, the *E. coli* isolates will be subjected to analysis using a "*m*ultiplex *p*olymerase *c*hain *r*eaction" (m-PCR) to detect if any of a diverse array of toxin genes are present. Such techniques are commonly employed to assess the epidemiology of infections caused by *E. coli*.

Note: If you are unfamiliar with the *p*olymerase *c*hain *r*eaction (PCR), watch the video located at the following URL: http://www.sumanasinc.com/webcontent/animations/content/pcr.html

The bacterial species, *E. coli,* is normally harmless to human health and is generally considered to be a commensal (ComEC). However, some strains do cause serious illness and are responsible for diarrheal diseases (i.e., diarrheagenic *E. coli* [DEC]). To date, pathogenic *E. coli* strains have been divided into seven distinct groups, five of which are DEC types. These five groups include entero-pathogenic (EPEC), entero-toxigenic (ETEC), entero-haemorrhagic (EHEC), entero-aggregative (EAEC), and entero-invasive (EIEC) strains. The virulence of DEC is often associated with toxin-encoding genes that promote pathogenesis.

Rapid diagnosis of diarrheal cases of *E. coli* infection are essential in the treatment and cure of afflicted individuals. Individuals at severe risk of death are those with persistent and unresponsive diarrhea, young children, older adults, and immunocompromised persons. Moreover, the proper characterization of the etiologic agent can be helpful in the proper treatment of these individuals as well as in the prevention of further infection.

Molecular-oriented methods have facilitated the increased rapidity and heightened specificity in identifying and characterizing DEC. This experiment employs a rapid and potentially cost-effective means for characterizing the different DEC groups based upon the toxin genes that each possesses. The method is based upon m-PCR, i.e., a DNA amplification method that uses a series of gene-specific primers in a single reaction tube. This method has been used successfully in several studies to identify DEC groups.

The m-PCR technique provides for concurrent amplification of various targets in a single tube rather than detection of each target using individual tubes (Fig. 1). m-PCR saves time and resources as well making possible the simultaneous comparison of multiple targets.

The experiment detailed herein shall utilize primers to amplify specific portions of toxin genes possessed by particular DEC groups as follows: EHEC (*stx1*, *stx2*, and *eaeA*); atypical EPEC

(*eaeA*) and typical EPEC (*bfp*); ETEC (*st* and *lt*); EIEC (*ial*); and EAEC (*eagg*). In addition, the presence of the *astA* gene, which codes for a heat-stable toxin in *E. coli*, shall be determined. As an internal control, all *E. coli* shall be identified by the amplification of a specific portion of the malate dehydrogenase gene (*mdh*). As an external control, primers will be used to amplify a portion of the *Klebsiella pneumoniae* glyceraldehyde 3-phosphate dehydrogenase gene (*gapdh*) which will be added to the reaction buffer. These primers do not amplify the homologous gene from *E. coli*. This control will serve to assess if substances released by the cells used as the DNA source during the m-PCR procedure inhibit target amplification.

Figure 1. Comparison of individual PCR and m-PCR. In the former (singleplex reactions), each tube contains one primer pair to amplify one specific target, whereas the m-PCR uses a number of different primer pairs to amplify multiple targets in one reaction. (Image Source: ThermoFisher Scientific; http://www.thermofisher.com).

Laboratory Safety Considerations

It is important to wholly recognize that this particular exercise shall include the handling of potential pathogens and other materials that may be hazardous. While chances of injury are very low, it is nonetheless essential that appropriate precautions be taken when warranted (e.g., wearing gloves, proper disposal of materials, caution with open flames, disinfection of work areas, etc.). Students are urged to ask questions should any portion of the following procedure not be clear, especially with regard to the handling and disposal of materials.

Important Techniques/Skill Sets

Students are *strongly encouraged* to review the following videos which demonstrate the proper use of the micropipette and the basic procedure involved in DNA gel electrophoresis.

Proper Use of a Micropipette. The proper use of a micropipette shall be critical to the success of this experiment. Students having no or limited prior experience with the proper operation of a micropipette should watch the video available through the following URL: https://www.labtube.tv/video/using-a-micropipet. Students may also wish to practice using the micropipette prior to initiating this experiment. The laboratory instructor can be a resource in helping students master this essential skill.

Performing Horizontal Gel Electrophoresis. The results of this experiment shall be visualized by horizontal gel electrophoresis of DNA fragments that you will generate. Students having no or limited prior experience with gel electrophoresis should watch the video available at the following URL: https://www.youtube.com/watch?v=vq759wKCCUQ.

Materials Required

The following materials are necessary to successfully conduct this exercise:

Organisms - The following organisms should be provided as 18-24 hour TSA slant cultures:

- *Escherichia coli*, isolated from the environment [abbreviated as ECENV]
- *Escherichia coli*, ATCC 25922 [abbreviated as *E. coli* 25922]
- *Escherichia coli*, strain ECTOIL (*astA*) [abbreviated as ECTOIL]
- *Klebsiella pneumoniae*, ATCC 13883 [abbreviated as *K. pneumoniae*]

Media and Reagents

Stored at 4°C (refrigerated)

- Tryptic Soy Agar (TSA) plates
- 3% TBE Wide Mini ReadyAgarose Precast Gels (15.6 x 10 cm, 20 wells, containing ethidium bromide; Cat No.1613030; Bio-Rad Laboratories, Hercules, CA)

Stored at -20°C (frozen)

- GoTaq® Green Master Mix (Cat. No. M7122; Promega Corp., Madison, WI)
- Reaction Primer Solution (RPS) [see Appendix A for details]
- 100-bp DNA size standard solution (in loading dye) [see Appendix B for details]
- *gapdh* stock solution (100 ng/µl) [This gene fragment was isolated from *Klebsiella pneumoniae*, ATCC 13883.]

Stored at Room Temperature

- Nuclease-free water, sterile in tubes
- Tris-Borate-EDTA Buffer (TBE, 1X concentration; 89 mM Tris, 89 mM boric acid, 2 mM EDTA)

Materials and Equipment

- Microfuge tubes (sterile, various colors)
  - 0.5 ml and 1.5 ml snap cap tubes
  - 200 µl snap cap (flat top ONLY) – also termed "PCR tubes"
- Microfuge racks (various sizes/capacities) to fit the microfuge tubes noted above
- Micropipettes (various volume capacities from 1 – 1000 µl)
- Filter-containing micropipette tips (various volumes to fit micropipettes)
- Disposable gloves (various sizes)
- Sterile toothpicks (flat ended)
- Vortex mixer
- Programmable thermal cycler (Model T100; Bio-Rad Laboratories, Inc.)

Note: pre-programmed as follows – 95°C for 15 min; 35 cycles consisting of 94°C for 45 s, 55°C for 45 s, and 68°C for 2 min; 72°C for 5 min; and 4°C indefinitely.

- Wide-Mini Sub Cell GT electrophoresis box (Model No. 1704468; Bio-Rad Laboratories, Hercules, CA)
- Electrophoresis power supply

Materials and Equipment (cont.)

- Ultraviolet light (UV) transilluminator
- SmartDoc Imaging System with E5001-590 filter (Model E5001-SDB; Accuris Instruments, Edison, NJ; http://www.accuris-usa.com/wp-content/uploads/2017/05/E5001-SDB-Smart-Doc-System-Manual-11-14-16.pdf)

Procedure

Students shall review and use the BIOL 3702L Standard Practices regarding the labeling, incubation, and disposal of materials.

This laboratory exercise shall be performed by groups of students per laboratory session. The laboratory instructor will direct how these groups will be formed. Preferably the groups will be established prior to starting this exercise. Hence, when a group meets for the first time, it is imperative that collaborative decisions be made such that each person in the group not only actively participates in this exercise, but also shall be responsible for one or more of the activities described below. Such assignments, however, do not relieve everyone in the group from fully knowing the details of this exercise and how the procedure is conducted.

To facilitate the active engagement of all individuals within a group, a "Group Duty List" has been appended to this laboratory exercise. Groups should gather together and make formal assignments to specific persons who shall be responsible for different portions of this exercise.

Preventing Contamination of PCR Materials

It is imperative to handle all materials in such a manner so as not to possibly contaminate PCR tubes, reagents, pipette tips, etc., with extraneous sources of DNA (e.g., do not touch tips or tops of tubes with your bare fingers). When using the micropipette, change pipet tips as much as practical to avoid cross contamination of reagents and solutions. To avoid introducing outside sources of DNA, some people wear disposable gloves, which shall be available. However, gloves are not really necessary if proper care is taken. Also, gloves may actually interfere with dexterity. By using common sense and careful handling of materials, DNA contamination can be avoided. PRACTICE PROPER HANDLING SKILLS!

Day 1

Streak each of the following bacterial strains on separate TSA plates:

- the laboratory *E. coli* [ATCC 25922] stock culture (negative genotype control strain);
- the *E. coli astA* [Strain ECTOIL] culture (positive genotype [toxin *astA*] control strain);
- the *E. coli* isolate (ECENV; unknown genotype) isolated from the prior experiment; and
- the *K. pneumoniae* stock culture [ATCC 13883] (non-*E. coli* negative genotype control).

Incubate the plates for 18-24 hours at 37°C.

Day 2

Note: Prior to performing the following steps, remove the following frozen items from storage and thaw at room temperature: RPS stock; GoTaq Green Master Mix; and *gapdh* stock solution. For short periods of time (< 1 hour), these reagents may be kept at room temperature or they may be kept on ice while in use. To save time and resources, small vials of these reagents may be provided either frozen or already thawed.

1) Prepare the PCR mixture by placing the following volume of each reagent into a sterile 0.5 ml microfuge tube: 2X GoTaq Green Master Mix (70 µl), RPS (14 µl), and nuclease-free water (28 µl). Mix the reagents well by pipetting up and down.

Note 1: In all pipetting steps, be sure to change the pipette tip each time an operation is completed. Do not cross contaminate reagents/samples! It is better to use an extra pipette tip than to make a mistake by trying to be scrupulous with resources.

Note 2: This volume of reaction mixture is prepared for seven samples, though only six will be tested – the four sample strains, a "no sample" (no DNA) control, and the *gapdh* external control only. The three *E. coli* strains among the samples (but not the *K. pneumoniae* strain) will also contain some *gapdh* DNA as an internal control. Any remaining reaction mixture accounts for small errors in pipetting, thereby assuring that each reaction contains the same volume. If more samples are to be tested, then this formulation should be adjusted accordingly.

2) Obtain six 200 µl PCR (flat top) tubes and label them on the lid #1 through #6.

Note: In addition to labelling, the use of different colored PCR tubes, if available, might be helpful in distinguishing one reaction tube from another.

3) To each of these tubes add 16 µl of the PCR mixture prepared in Step 1.

Note: At this point, there should be a small amount (in theory, 16 µl) of reaction mix remaining. This may be discarded in the appropriate receptacle.

4) In the PCR tubes #1, #2, #3, and #5, add 1 µl of the *gapdh* solution.
5) Transfer 1 µl of nuclease-free water in the PCR tube #4.
6) Transfer 3 µl of nuclease-free water in the PCR tube labelled #5. This will serve as the external (*gapdh*) control. Be sure to close the lid of this tube securely.
7) Transfer 4 µl of nuclease-free water in the PCR tube #6. Be sure to close the lid of this tube securely.
8) Obtain four sterile 1.5 ml microfuge tubes.
   a) Label one sterile 1.5 ml microfuge tubes as #1 (this tube will receive cells from *E. coli* ATCC 25922].
   b) Label a second sterile 1.5 ml microfuge tubes as #2 (this tube will receive cells from *E. coli E. coli astA* culture [Strain ECTOIL]).
   c) Label a third sterile 1.5 ml microfuge tubes as #3 (this tube will receive cells from the student environmental *E. coli* isolate [strain ECENV]).
   d) Label the fourth sterile 1.5 ml microfuge tubes as #4 (this tube will receive cells from *K. pneumoniae* ATCC 13883).
   e) Aseptically add 100 µl of nuclease-free water to each of the labeled tubes. Close the lid of each tube.

Note: In addition to labelling, the use of different colored 1.5 ml microfuge tubes, if available, might be helpful in distinguishing one tube from another.

9) Take the 1.5 ml microfuge tube labelled #1 and open the lid. From the streak plate prepared on Day 1 for the laboratory *E. coli* stock culture [ATCC 25922], use a sterile toothpick to remove a single, isolated colony (approximately 1 mm in diameter; if the colonies are smaller, pick two colonies) from the respective TSA plate and place the toothpick in the water contained in the microfuge tube labelled #1. Rotate the toothpick rigorously back and forth to remove the colony/colonies of cells. Remove the toothpick and discard it in the

appropriate container. Close the microfuge tube lid securely. Using a vortex set at the highest speed, mix the contents of the tube for 5-10 seconds to thoroughly suspend the bacterial cells.

10) Similarly, repeat step 9 for each of the remaining streak plates prepared on Day 1 and prepare cell suspensions from the *E. coli astA* culture [Strain ECTOIL], the environmental *E. coli* isolate, and the *K. pneumoniae* culture [ATCC 13883] in the 1.5 ml microfuge tubes labelled #2, #3, and #4, respectively.
11) Place 3 µl of the cell suspension from 1.5 ml microfuge tube #1 to the PCR tube labelled #1.
12) Similarly, repeat step 11 for each of the remaining cell suspensions prepared in Step 10 and place 3 µl of the cell suspension prepared from the *E. coli astA* culture, the environmental *E. coli* isolate, and the *K. pneumoniae* culture in the PCR tubes labelled #2, #3, and #4, respectively.
13) Place all six of the PCR tubes in the thermal cycler being certain that the lids are properly and snugly closed. To initiate the amplification reaction, follow the instructions available at the following URL: http://crcooper01.people.ysu.edu/microlab/thermocycler.pdf.

    The total time for completion of the amplification cycles is about 3 hours. Return as soon as possible to retrieve the samples and shut down the thermal cycler using the instructions provided (http://crcooper01.people.ysu.edu/microlab/thermocycler.pdf). Store your samples at 4°C (overnight or up to two days) or -20°C (longer than two days) until the gel analysis (Day 3) is conducted.

Day 3

The following protocol employs the Wide Mini Sub Cell (Bio-Rad Laboratories, Inc.) for gel electrophoresis of the DNA fragments generated by the above PCR. The protocol also uses pre-poured 3% agarose gels containing ethidium bromide made specifically for this gel box. Because the gel contains ethidium bromide, a known mutagen, it is important to handle the gel and the post-run buffer while wearing gloves. When this exercise is completed, properly dispose of all materials (gels, gloves, buffer, etc.) as directed by the laboratory instructor.

Note: Be sure to review the video at https://www.youtube.com/watch?v=vq759wKCCUQ. This video provides some essential details on how to perform gel electrophoresis, particularly the critical step of loading samples into the wells. It might be appropriate to choose someone from the group who has steady hands and good micropipetting technique to load samples in the gel.

1) Set the gel box on a level surface and carefully remove the lid. Carefully remove the pre-poured agarose gel from its packaging being sure to remove the plastic cover.
2) The gel is supported by a plastic tray containing well markings and a measuring scale on one side. The plastic support at the "bottom" of the gel has two small protruding tabs. These shall help to orient and secure the gel in the electrophoresis box. Gently place the gel on the platform within the electrophoresis box with the tabs on the plastic gel support nearest positive (red) pole and the wells nearest the negative (black) poll.
3) Next, gently pour about 650-700 ml of TBE buffer into the gel box. The level of the buffer should cover the surface of the gel by 2-4 mm in depth, but the level should be no higher than the black line indicator on the side of the gel box.
4) If not thawed, allow the 100-bp DNA ladder/dye standard to come to room temperature. Carefully pipette 10 µl of the 100-bp DNA ladder/dye standard into the wells numbered 1 and 7 of the agarose gel.

5) Into well numbers 2 through 6, respectively, sequentially add 20 µl of the PCR sample from tubes #1 through #5.

Note 1: Agarose gel loading dyes already constitute a portion of the GoTaq Green Master Mix.

Note 2: The pre-made agarose gel has twenty available lanes. Hence, a second group can also load their samples on the gel using the remaining lanes, thereby saving resources.

6) Replace the lid on the gel chamber with the terminals correctly positioned to the matching electrodes, i.e., black to black and red to red. Connect the electrodes to the power supply again being certain that the electrodes match the terminals on the power supply, i.e., black to black and red to red.

7) **In this procedural step, use extreme caution with this source of high-voltage electricity.**

Switch on the power supply and set the voltage to 105 volts (with a range of 100-115 volts, but no more than115 volts). At this point bubbles should be able to see coming from the wires at each end of the gel box. If not, there may be an additional on/off switch on the power supply or the cable connections are not properly established. Notify the laboratory instructor if there are problems.

The migration of the samples from the wells into the gel should be visible within minutes. The various dyes should move from the negative (black) electrode towards the positive (red) electrode. If the dyes are flowing in the opposite direction (towards the negative [black] pole), turn off the power supply and immediately notify the laboratory instructor.

Note 1: The style and set up of each power supply often differs. Be sure that the voltage is being set and not ampage!

Note 2: The voltage chosen for electrophoresis is dependent upon the equipment and the buffer used. Typically, voltage used for electrophoresis varies in magnitude from 1 to 8 V/cm (the distance between the electrode wires). The conditions noted above work well for the buffer, the size of the gel box chosen for this particular exercise, and the brand of gel box being used. However, though the rate of DNA migration in the gel can be slowed by using a lower voltage, it is not advisable to use a voltage that goes beyond V/cm.

8) Allow the electrophoresis to continue for 1.5 to 2 hours or until the first dye line (yellow) in the sample lanes almost reaches the end of the gel. At this time, turn off the power supply.

Note: To have the DNA migrate the same distance as the above parameters, but for a longer period of time, the following general formula should be followed:

(105 Volts) x (2 hours) = (Voltage to use) x (Desired time of electrophoresis)

Hence, if a gel were to run for the same DNA migration distance as above, but over 6 hours instead of 2 hours, the answer would be calculated as follows:

(105 Volts) x (2 hours) = (Voltage to use) x (6 hours)

210 V/hours = (Voltage to use) x (6 hours)

5 V = Voltage to use

Therefore, if 35 V were used instead of 105 V as above, it would take *approximately* 6 hours for the DNA to migrate to the same distance.

This information can be used if it is inconvenient to return to process the gel after 2 hours.

9) Clean the glass surface of the UV transilluminator using a small amount of water and a KimWipe™. Do not use a paper towel or other non-soft wipe. Wipe the surface dry, then place the rubber UV-blocking frame on it.

10) Remove the electrode connections to the power supply, then carefully remove the lid of the gel box. Wearing gloves, lift the gel tray from the buffer and allow the excess liquid to drain back into the gel box being careful to not let the agarose gel slide off the tray. (Place a finger or two on the side of the gels to prevent this.) Position the gel squarely within the rubber frame on the UV transilluminator using care to eliminate any bubbles underneath.
11) Visualize the electrophoresis results using the SmartDoc Imaging System (Fig. 2; Accuris Instruments). Brief instructions are presented below.

Note: Complete information can be found in the SmartDoc™ 2.0 instruction manual (http://www.accuris-usa.com/wp-content/uploads/2017/05/E5001-SDB-Smart-Doc-System-Manual-11-14-16.pdf). A video demonstrating the use of a smartphone with the SmartDoc Imaging System can be found at the following URL: https://youtu.be/_CAGuRHG7Uw. The relevant portion begins at 2:22 minutes into the video.

a) Position the SmartDoc™ imaging enclosure over the agarose gel aligning it with the opening of the rubber frame.
b) Insert the E5001-590 filter into the square slot on the top platform. The side with the Accuris logo should be facing up.
c) Select the camera mode on the smartphone and turn off the flash setting.
d) Place a smartphone face down onto the top platform aligning the camera lens with the filter. When properly positioned, the gel will be seen in the device's display screen.
e) Focus on the gel image as required. The zoom function of the smartphone's camera phone can be used to enlarge the gel image in the display, but the resolution of the image may decrease.

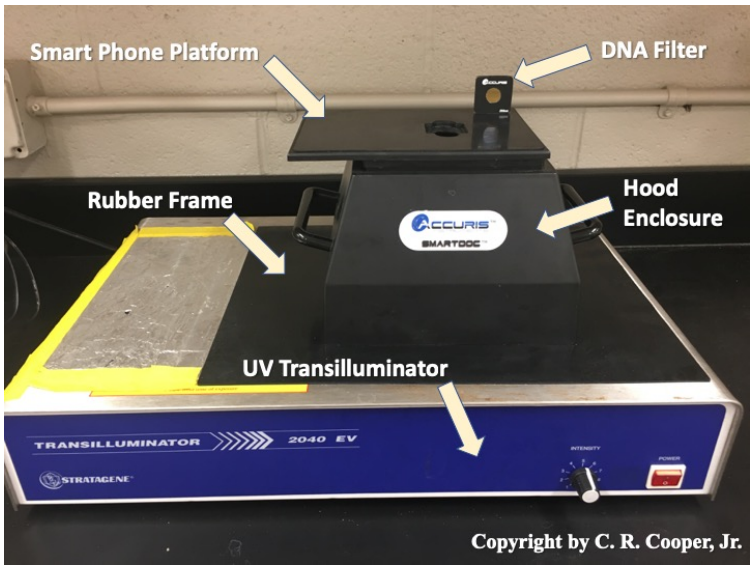

Figure 2. SmartDoc™ Imaging System. A rubber frame is placed on the glass surface of a UV transilluminator. This frame blocks out extraneous visible and UV light as well as marks off an area on the glass surface. Within the framed surface, a DNA gel stained with ethidium bromide has been placed. The hood enclosure is then positioned over the gel and within the area delimited by the rubber frame. The DNA filter (590 nm) is set 'logo up' within the square slot in the smart phone platform. The camera lens of a smart phone is positioned over the lens, the transilluminator turned on, and an image can now be visualized and photographed.

f) Take one or more photographs of the gel being sure to capture all the relevant lanes. Typical results are shown in Fig. 3 (next page).

Record your observations on the report sheets attached to this exercise being sure to include the gel photograph.

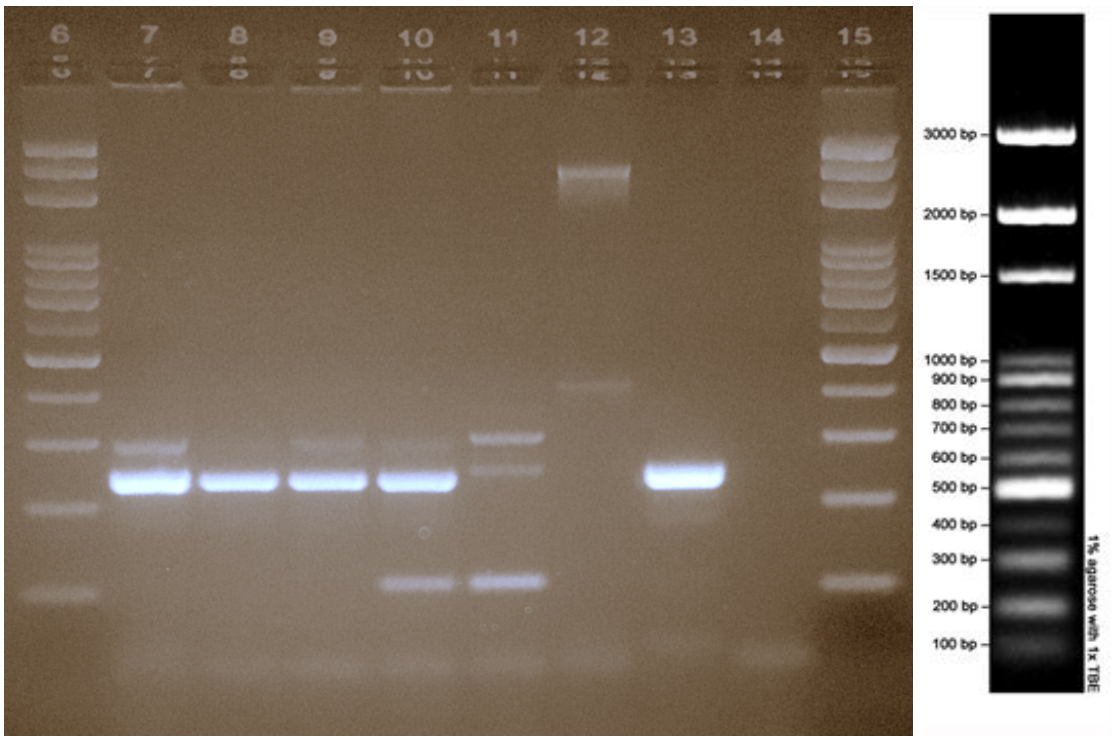

Figure 3. Right Image - Typical m-PCR results generated by following the protocol detailed in this exercise. Lanes 6 and 15: 100 base pair ladder (see ; Lane 7: *E. coli* ATCC 25922; Lane 8: environmental *E. coli* isolate YSU 91417; Lane 9: *E. coli* isolate from human urine (YSU ECUR); Lane 10: *E. coli* isolate from toilet water (ECTOIL); Lane 11: *K. pneumoniae* ATCC 13833; Lane 12: *Klebsiella oxytoca* ATCC 43131; Lane 13: *gapdh* external control; Lane 14: no DNA control. Left Image – Size indications of 100 base pair ladder used in the right image (Image Source: http://www.amresco-inc.com/DNA-MW-MARKER-100BP-LADDER-K180.cmsx). See the text of this exercise for possible interpretations of these results.

Possible Interpretations of Results in Figure 3: [*The following is solely an example interpretation and should not be plagiarized*. This interpretation is based upon the tables presented in the publication by Omar and Barnard (2014) *Detection of diarrhoeagenic Escherichia coli in clinical and environmental water sources in South Africa using single-step 11-gene m-PCR*. *World Journal of Microbiology and Biotechnology* 30: 2663–2671. Note that additional references were cited in this interpretation.]

Lanes 7 through 11 indicate that all the organisms subject to m-PCR possessed a 300-base pair (bp) product (faint in some lanes) and another at approximately 254 bp. The former is likely the *mdh* gene fragment detailed previously (https://www.ncbi.nlm.nih.gov/pubmed/24969140 indicating that the PCR was successful. The 254 bp band is equivalent in size to that in Lane 13 in which the DNA source was a fragment of the *gapdh* gene. The presence of this PCR product in Lanes 7 through 11 indicates that the cellular constituents of the source organisms did not interfere with the amplification reaction. No PCR products were noted in the "no DNA" control (Lane 14) indicating that no extraneous contamination occurred in the reactions. Lane 12 contains PCR products generated from *Klebsiella oxytoca*. The one band is far too great in size (approximately 2000 bp) to be a likely gene product targeted by this protocol, but one band is about 400 bp in size and may possibly represent the *bfp* gene. The *bfp* gene family encodes

pili involved in host-cell attachment (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4810605/). Significantly, Lanes 10 and 11 contain a PCR product slightly greater than 100-bp in size. Lane 10 represents an *E. coli* strain that possesses the *astA* gene. This gene encodes a heat-stable toxin involved in diarrheal disease (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3811233/). The 100-bp band is likely a fragment of this gene. Curiously, *K. pneumoniae* appears to possess the same gene suggesting that under the appropriate conditions that this strain could cause significant morbidity. The very faint bands below 100 bp in size are excessive primers in the reaction mix and not PCR products.

****

Appendix A: Reaction Primer Solution (RPS)

Primers sequences can be found in the publication by Omar and Barnard (2014) at the following URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4150989. Primers should be ordered from a reliable vendor and stock solutions of each should be made in nuclease-free water to a final concentration of 100 µM.

Reaction Primer Solution (RPS) is prepared by pipetting the following volumes of the various primers (stock solutions of 100 µM) in a sterile 1.5 ml microfuge tube containing 440 µl of nuclease-free water:

Primers	Volume	RPS Conc.	Final Reaction Mix Conc.
mdh-F and *mdh*-R	10 µl each	1.0 µM	0.1 µM
ial-F and *ial*-R	20 µl each	2.0 µM	0.2 µM
eaeA-F and *eaeA*-R	30 µl each	3.0 µM	0.3 µM
bfpA-F and *bfpA*-R	20 µl each	2.0 µM	0.2 µM
eeag-F and *eeag*-R	20 µl each	2.0 µM	0.2 µM
stx1-F and *stx1*-R	50 µl each	5.0 µM	0.5 µM
stx2-F and *stx2*-R	30 µl each	3.0 µM	0.3 µM
lt-F and *lt*-R	10 µl each	1.0 µM	0.1 µM
st-F and *st*-R	50 µl each	5.0 µM	0.5 µM
astA-F and *astA*-R	20 µl each	2.0 µM	0.2 µM
gapdh-F and *gapdh*-R	20 µl each	2.0 µM	0.2 µM

Appendix B: DNA Size Standard

Prepare the DNA size standard by pipetting 30 µl of the 100-bp DNA ladder standard (Cat. No. K180; Amresco, Inc., Solon, OH) into a 1.5-ml microfuge tube. To this, add 6 µl of the agarose gel loading dye (Cat. No. E190; Amresco, Inc.) and gently mix. This solution can be stored on ice (4°C) or at room temperature when not in immediate use, but for long-term storage the tube should be place at -20°C.

For use in gel electrophoresis, add 6 µl of the DNA standard solution to a well.

GROUP DUTY LIST

Prior to/during Day 1

Confirm Isolate as *E. coli*: _____

Day 1

Streaking Isolates: _____

Day 2

Prepare PCR Mixture: _____

Set Up Reactions: _____

Preparing Cell Suspensions: _____

Transfer Cells to Reaction Tubes: _____

Run Thermal Cycler: _____

Retrieve Samples from Thermal Cycler: _____

Day 3

Set Up Gel Box: _____

Load Samples into Gel: _____

Perform Electrophoresis: _____

Visualize/Document Results: _____

M-PCR LABORATORY REPORT

Date: _____ [Only one report per group is to be submitted]

Group Member Names (please print):

[NOTE – if a member of a group does not actively participate in this exercise, do not put their name on this list!]

_____

_____

_____

Source of Environmental Isolate: _____

Attach Gel Picture and Complete the Legend Below:

Lane	Sample Type
1	_____
2	_____
3	_____
4	_____
5	_____
6	_____
7	_____
8	_____

Results, Discussion, Conclusions: Based upon the tables presented in the publication by Omar and Barnard (2014) (https://www.ncbi.nlm.nih.gov/pubmed/24969140), use the attached sheet (next page) to interpret your gel results and determine the genotype and phenotype of your environmental *E. coli* isolate. Make certain to discuss results of each lane, explain any anomalies or errors that affected your results, and the possible implications of your data. Use outside references, if relevant, but cite them by electronic (URL) links rather than full citations. Be concise, yet complete in your response. Critical thinking and grammar shall count towards the final scoring of this section of this laboratory report.

Note: *Submit only ONE COPY of the report pages - NOT those of the protocol – which MUST also include a copy of the gel photo.* This single copy will represent the report for the entire group. *YOU MUST STAPLE* both pages together in the upper left-hand corner. Everyone in your group will receive the same score for this laboratory report. Work collectively, effectively, and collegially.

Group Member Names (please print):

_____

_____

_____

Experimental Results, Discussion, Conclusions: