Isolation and Characterization of *Escherichia coli* from Animals, Humans, and Environment

research-article

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Abstract

Working on a diverse species of bacteria that have hundreds of pathotypes representing hundreds of strains and many closely related family members is a challenge. Appropriate research design is required not only to achieve valid desired outcome but also to minimize the use of resources, including time to outcome and intervention. This chapter outlines basics of *Escherichia coli* isolation and characterization strategies that can assist in research designing that matches the set objectives. Types of samples to be collected, collection and storage strategies, and processing of samples are described. Different approaches to isolation, confirmation and concentration of various *E. coli* strains are summarized in this chapter. Characterization and typing of *E. coli* isolates by biochemical, serological, and molecular methods have been explained so that an appropriate choice is made to suite a specific *E. coli* strain/pathotype. Some clues on sample and isolate preservation for future use are outlined, and general precautions regarding *E. coli* handling are also presented to the researcher to avoid improper planning and execution of *E. coli*-related research. Given different options, the best *E. coli* research design, however, should try as much as possible to shorten the length of time to outcomes.

Keywords: *E. coli*, β-glucuronidase, Enterobacteriaceae, cryoprotectant, IMViC

1. Introduction

Escherichia coli is Gram-negative, facultative anaerobic, and rod-shaped bacterium of the genus *Escherichia*. This is a large diverse group of bacteria commonly found in the lower intestine of

warm-blooded organisms. Most of them are commensals inhabiting the lower gastrointestinal tract (GIT) of mammals. The other strains that are pathogenic are categorized into two groups, according to the site of infection. *E. coli* that infect and cause disease syndromes in the gastrointestinal tract are intestinal pathogenic *E. coli* (IPEC). Those that cause disease syndromes in systems other than gastrointestinal tract are called extra-intestinal *E. coli* (EXPEC). The commensal group form part of gut microbiota and is used as indicator bacteria for fecal contamination.

Pathogenic *E. coli* group consist of many strains, which for simplicity, can be grouped according to the virulence factors they possess or pathological effects they cause. The intestinal pathogenic *E. coli* include enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and verocytotoxigenic *E. coli* (VTEC) according to O'Sullivan et al. [24]. Extra-intestinal pathogenic *E. coli* includes uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), and sepsis-causing *E. coli* (SEPEC) [1].

Most pathogenic *E. coli* are transmitted by fecal-oral route from food materials, water, animals, and environment. Depending on the pathotype and the system, *E. coli* infection may cause a range of syndromes including watery, mucoid, or bloody diarrhea; abdominal cramps; urinary tract infection syndromes; and meningitis. Complications to pathogenic *E. coli* infection may lead to hemorrhagic uremic syndrome (HUS). These syndromes have been reported as food poisoning outbreak, travel-related illness, or animal or contaminated environment contact-related diseases. Global *E. coli*-related morbidities and mortalities are high. The estimates for the year 2010 show that there were 321,969,086 cases of *E. coli* food-borne illness which is 16.1% of global food-borne diseases. Also there were 196,617 deaths attributable to *E. coli*-related food-borne poisoning which is 0.02% of global mortalities due to food poisoning [2]. This situation calls for regular and continuous investigations to diagnose, treat, and prevent *E. coli*-related diseases.

Inappropriate planning of research due to lack of knowledge may lead to undesired outcomes. For instance, if one aims at assessing the magnitude of shading of diarrheagenic *E. coli* in cattle feces, he or she may end up with underestimated results if he or she chooses to use sorbitol MacConkey agar as a screening media because not all diarrheagenic *E. coli* are sorbitol fermenters. Likewise, if one is looking for *E. coli* O157:H7 in a sample, the use of media that discriminate bacteria according to the presence of β-glucuronidase activities may lead to missing the desired outcome since *E. coli* O157:H7 do not possess such an enzyme. This chapter, therefore, outlines approaches to isolate and characterize *E. coli* from animals, humans, and the environment so that planning and implementation of *E. coli*-related research can match the set objectives and desired outcome.

2. Collection and storage of sample for *E. coli* isolation

2.1. Sample collection

E. coli predominantly inhabit the gastrointestinal tract of mammals and are shed to the environment through feces. The feces from mammals can be collected for the purpose of *E. coli* isolation. In this case, fresh fecal material from individual humans or animals can be used. Dry or sunburnt fecal samples may lead to false negative results. Shading of *E. coli* in feces makes this

microorganism abundantly available in the environment. As a result, *E. coli* can be recovered from water, soil, contaminated food material, and surfaces.

Sampling of the soil for isolation of *E. coli* requires taking the sample 2–5 cm beneath the surface. Top soils may contain dead bacteria. Water samples can be collected for *E. coli* detection. *E. coli* can also be isolated from contaminated surfaces of both animate and inanimate materials. Animate surfaces include human or animal body surface. Food surfaces or working structures such as table, knives, and clothes can be a good source of *E. coli*. Food surfaces such as meat, eggs, or fish can be used to isolate *E. coli*, depending on the objective of the study. Animal fecal sample can be taken from the rectum (large animals) or fresh droppings can be collected by fingers of a gloved hand. Human stool can be put in a container with a stopper. Water samples can be collected by different methods according to nature of the water body. Still surface can be collected by hand deep method, whereas flowing water sample collection requires depth-and-width-integrating methods. In this type of water body, for example, a stream, 5–10, or more samples are collected across the vertical depth and width [3]. Samples from surfaces such as hide, table, knife, and the likes can be obtained by sweeping a buffered peptone water with premoistened swabs or sponge on the sampling surface in a Z-pattern [4]. The sponge or swabs that covers approximately 400–1000 cm^2 are then put in 100 ml of tryptic soya broth for further processing.

2.2. Sample storage

Samples for *E. coli* isolation are best processed right after collection, normally within 24 h. This includes inoculation into enrichment or inoculation onto solid culture media. When situation does not allow, a sample can be stored at low temperatures that restrict further cell division, but at the same time, allows survival of the bacteria. Surface water samples for *E. coli* isolation stored at below 10°C, but not freezing, can give comparably good results for up to 48 h after collection [5].

Sometimes analysis of fecal samples immediately after collection is impractical due to temporal and spatial challenges or assessment of old samples can be a requirement. In this case, fecal/stool samples should be stored for later laboratory isolation or old samples that were appropriately stored are recalled. Fecal samples will maintain *E. coli* population density, clonal characteristics, and diversity as fresh samples when stored in glycerol broth at lower temperatures than −70°C for 30 days up to 1 year. The fecal sample may form 10% of final concentration in 10% glycerol broth. However, storage of this sample at −20°C for the same time period will lead to a decrease in bacteria population density but increased diversity [6, 7]. Moreover, samples stored in glycerol broth will have more similar *E. coli* isolates to isolates from the fresh original sample than those from samples stored without mixing with glycerol, and if samples are repeatedly thawed, then addition of glycerol broth is recommended. Pure samples stored for a long time without glycerol lead to decrease in *E. coli* number [6]. Therefore, longer storage of fecal samples without appropriate processing may lead to inaccurate results.

3. Isolation of *E. coli* and quality control

3.1. Isolation of *E. coli*

Different options are available for the isolation of *E. coli*. The choice depends on target strain and objective of isolation. The ability to ferment lactose gives an option to use MacConkey agar to discriminate *E. coli* from other nonlactose fermenting coliforms from fecal, stool, food, water, and soil samples. Sample suspension (for solid samples) is made at any concentration, for example, 5% in normal saline or phosphate buffer solution and inoculated onto MacConkey agar followed by 18–24 h incubation at 37°C. Pink, round medium-sized colonies are picked as *E. coli* suspect colonies. All *E. coli* strains can be captured on MacConkey agar, and this approach gives a wide spectrum of strains to work on. Incubation of inoculated culture media at 45°C selects for thermophilic *E. coli* strains.

The concentration of sample suspension may be set at different levels such as 1 g of solid sample in 19 ml of normal saline or phosphate buffer solution (5%), 1 g in 9 ml (10%) or 1 g in 4 ml of diluent (20%). However, the concentration of sample suspension will affect the number of colonies on the culture plate. This is well evidenced in bacteria count procedures whereby higher dilution, like 10^5, will give lower number of bacteria than low dilutions, for example, 10^1. This is because the bacteria growth rate depends on initial cell density in the sample [8].

Sample suspension can be enriched by 24 h incubation at 37°C in nondifferential broth such as Muller-Hinton or nutrient broth. This procedure will allow multiplication of *E. coli* and hence increase the chance of *E. coli* isolation especially when infrequent strains, such as pathogens, are the target. The generation (doubling) time for *E. coli* at 37°C incubation is 17–18 min [8], therefore, in 18–24 h incubation there will be 60–80 *E. coli* cell generations. However, clonal variability will decrease when samples are enriched because same bacteria increase in number. Therefore, this procedure is suitable when the research aims at a mere presence of a single specific strain and not its variants.

The weight of the sample and the volume of diluent used in making the sample suspension may affect the probability of bacteria recovery. Large sample weight normally increases the sensitivity of the isolation procedure. For example, in *E. coli* studies to isolate nonsorbitol-fermenting Shiga toxin-producing *E. coli* (NSF STEC) whereby *E. coli* broth was used to enrich fecal samples, different prevalence measure was obtained. When 10 g of sample was suspended in 90 ml of *E. coli* broth, the prevalence of Shiga toxin-producing *E. coli* (STEC) obtained was 1.3% [9], while the suspension of 20 g in 180 ml of same diluent resulted into a prevalence 11.1% NSF STEC [10].

Purification of *E. coli* colonies can be done in nondifferential media such as blood or nutrient agars. Depending on the degree of colony density, a series of inoculations can be desired until pure, single, or solitary colonies are obtained.

3.2. Quality control

These are procedures undertaken to validate the accuracy of the bacteria isolates. Among the measures of quality control in isolation of *E. coli* include incubation of uninoculated media plates at 37°C overnight. The media plates should have no microbial growth after incubation. This will ensure that the isolates obtained after inoculation come from the samples and not due to contamination. Moreover, uninoculated media plate should be incubated simultaneously with

inoculated media plates. Use of reference positive controls strains, e.g.*E. coli* ATCC 25922, will also help to ensure the isolates are the targeted bacteria.

For water samples, quality control measures may involve the use of blank and sample replicates. The true samples and the blanks are simultaneously incubated. The blank sample will tell that the sampling equipment has not been contaminated. The replicate results will assess the presence of variation for which explanations should be sorted out.

4. Confirmation of *E. coli* isolates

Confirmation of *E. coli* isolates can be done by biochemical, enzymatic, or molecular methods. The choice of the method depends on many factors including availability of resources. The confirmation methods include biochemical methods, such as IMViC and Analytical Profile Index 20E (API 20E) systems, enzymatic methods, for example, use of brilliance *E. coli* agar or Petrifilm Select *E. coli* count plate, and molecular techniques such as MALD-TOF.

4.1. IMViC tests

E. coli isolates can be confirmed biochemically by the use of a traditional method called IMViC tests. This is a set of four tests that are used to differentiate members of the family Enterobacteriaceae. IMViC is an abbreviation that stands for the Indole, Methyl red, Voges-Proskauer, and Citrate utilization tests. In Indole test, the bacteria are tested for their ability to produce indole from tryptophan (amino acid) using the enzyme tryptophanase.

The indole reacts with the aldehyde in the Kovac's reagent to give a red or a pink ring at the top of the tube. Peptone water in a tube, which contains tryptophan, is inoculated with bacteria isolate to be tested. The mixture is incubated overnight at 37°C. Then, a few drops of Kovac's reagent are added to the mixture and formation of a red or a pink colored ring at the top is a positive reaction. *E. coli* are indole-positive bacteria.

Methyl red test detects the ability of a bacterium to produce acid from glucose fermentation. Methyl red, a pH indicator, remains red in color at a pH less or equal to 4.4. The bacterium to be tested is inoculated into glucose phosphate (MRVP) broth, which contains glucose and a phosphate buffer and incubated at 37°C for 48 h. Three to five drops of MR reagent are added to the tube. Red color development is a positive reaction that occurs when the bacteria have produced enough acid to neutralize the phosphate buffer. Yellow discoloration occurs to MR-negative bacteria. *E. coli* are MR-positive bacteria.

Voges-Proskauer test is used to detect the presence of acetoin in the bacteria-containing media. Acetoin is oxidized to diacetyl in the presence of air and sodium hydroxide. Diacetyl, in the presence of alpha-naphthol, reacts with guanidine to produce red color. In order to perform VP test, the test bacterium is inoculated into glucose phosphate (MRVP) broth in a tube and incubated for 72 h.

Addition of 15 drops of alpha-naphthol to the test broth is followed by shaking. Then add five drops of 40% potassium hydroxide (KOH) to the broth and shake well. Allow the tube to stand for

15 min to see a positive red discoloration, after 1 h of no color change the isolate is categorized as VP negative. *E. coli* is VP negative.

Citrate utilization test detects the ability of the bacteria to use citrate as its sole source of carbon and energy. Citrate agar media contains a pH indicator called bromthymol blue. The agar media changes from green to blue at an alkaline pH. Streak a loopful of bacteria onto a citrate agar slant without stabbing the butt and incubate at 37°C for 24 h with a loose cap. Citrate in the media breaks down to oxaloacetate and acetate due to action of an enzyme citritase. Oxaloacetate is further broken down to pyruvate and CO_2. Production of Na_2CO_3 from sodium citrate changes the media into alkaline pH, and hence color change from green to blue. Blue color formation is a positive reaction, whereas the slant remaining green colored is a feature for negative test. *E. coli* is citrate negative.

This conventional IMViC test method gives results (**Table 1**) that are similar to an agar plate IMViC method [11]. *E. coli* and *Proteus vulgaris* show the same IMViC pattern, but *Proteus* spp. are lactose-negative, motile, and show swarming behavior.

Bacterium	**Indole**	**MR**	**VP**	**Citrate**
Escherichia coli	+	+	−	−
Klebsiella pneumoniae	−	−	+	+
Enterobacter aerogenes	−	−	+	+
Salmonella species	−	+	−	+
Shigella species	−	+	−	−
Proteus vulgaris	+	+	−	−
Proteus mirabilis	−	+	−	+
Citrobacter freundii	−	+	−	+

Table 1.

MViC test results of some members of family Enterobacteriaceae (Adapted from Powers and Latt [11]).

4.2. The API 20E system

Analytical Profile Index 20E is a set of biochemical tests specific for differentiating between members of the Gram-negative bacterial family Enterobacteriaceae. It is used for rapid identification of already known bacteria. API 20E system is made up of 20 small reaction tubes that contain dehydrated substrates for detection of the enzymatic fermentation of sugars by the test isolates. This fermentation occurs during incubation, and the resulting pH change is detected by an indicator. It is important to confirm that the test culture is of an Enterobacteriaceae first, by doing a quick oxidase test. Enterobacteriaceae are oxidase negative.

Inoculate the suspension of a pure culture into each of the 20 reaction tubes and Incubate the tray at 37°C for 18–24 h. You can read the color change in some compartments right after incubation,

but some may require additional reagents. Mark each test as positive or negative on the lid of the tray and score them. Add up the scores, the maximum score being seven, to get a 7-digit code that is used to identify the bacteria by using the online database.

4.3. Enzymatic activities

Strict selective media that check for specific enzymatic activities in *E. coli* can be used to confirm *E. coli* isolates. For instance, brilliance *E. coli* agar or Petrifilm Select *E. coli* count plate can be used to check for presence and activity of β-glucuronidase enzyme. Beta-glucuronidase enzyme, which is specific to *E. coli*, cleaves glucuronide substrate resulting in purple and blue-green colonies in Brilliance *E. coli* agar and Petrifilm *E. coli* Select count plates, respectively. Non-*E. coli* coliforms have ß-galactosidase only, which enable them to break down lactose, whereas most of *E. coli* have both β-galactosidase and β-glucuronidase. However, *E. coli* O157 are glucuronidase negative; therefore, these media are not appropriate for initial screening of *E. coli* population but can be used to differentiate *E. coli* O157 from confirmed *E. coli* population.

4.4. MALD-TOF mass spectrometry

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALD-TOF) mass spectrometry is a rapid and accurate method for microorganism identification. Principally, the biomolecules are allowed to gain or lose electrons (ionization) and then sorted based on their mass to charge ratio, when subjected to electric or magnetic field. The spectrum generated is analyzed and compared to stored profiles using software. These spectra, which are species-specific, can be used to confirm microorganism, for example, *E. coli* or discriminating closely related species such as *E. coli* and Shigella.

5. Convergence of *E. coli* isolates

E. coli form a large diverse species of bacteria that is difficult to handle when targeting a specific strain. Working on any *E. coli*-suspect colony from less discriminatory procedures prolong the time interval to isolate confirmation and utilizes more resources. The use of selective media helps to achieve this goal, but only when target *E. coli* strain is well defined. Otherwise, other approaches should be employed. Selection of *E. coli* isolates with some common features may be used to narrow down the population size. Converging similar *E. coli* isolates can be done by employing different procedures that utilize antimicrobial resistance, enzymatic, and immunogenic reactions and genetic characteristics of specific *E. coli* to mention a few. The choice of the method of converging *E. coli* isolates depends on different factors including study objectives, bacteria characteristics, skill, and resource availability.

Resistance to a single antimicrobial agent or combined resistance to more than one antimicrobial agent can be used to get *E. coli* isolates with common features. Resistance to common antimicrobial agents used in an area can be used to screen *E. coli* isolates before further analyses. For example, in a study to assess genetic similarities between *E. coli* isolates from humans, cattle, and the environment, Lupindu and his colleagues [12] chose to isolate *E. coli* with resistance to tetracycline and ampicillin to concentrate *E. coli* from the general population. *E. coli* isolated from MacConkey agar was subjected to ampicillin-tetracycline solution using Petrifilm Select *E. coli*

count (SEC) plate. Out of 1046 *E. coli* isolated from MacConkey agar, 118 isolates were resistant to ampicillin-tetracycline drug combination. Antimicrobial stock solution and bacteria inoculation were executed as previously described in Ref. [13]. One milliliter of antimicrobial stock solution containing 0.32 mg ampicillin and 0.64 mg was placed on the bottom lid. After 2 h of absorption of the antimicrobial solution, 2 μl of standardized sample suspension was spot-inoculated onto the antimicrobial embedded lower lid of Petrifilm SEC plate. The upper lid was closed after 10 min, and the plate incubated at 42°C for 24 h. Round, medium-sized *E. coli* colonies appear dark-green due to the presence of β-glucuronidase activity on glucuronide substrate in indicator embedded medium. This procedure was also used to confirm the *E. coli* isolates that were further analyzed by PFGE for their genetic relatedness.

Ability of some *E. coli* strains to ferment different sugars can be used to concentrate strains of interest. All *E. coli* are lactose fermenters, but only some can ferment sorbitol. Sorbitol (instead of lactose) is mixed with MacConkey agar to form sorbitol MacConkey agar. This media can be used to discriminate sorbitol fermenting *E. coli* from nonsorbitol fermenters (NSF) and hence narrow down the *E. coli* population to a group of interest. The most common pathogenic *E. coli* that can be targeted by this procedure is *E. coli* O157:H7. Majority of *E. coli* O157:H7 and a few other diarrheagenic *E. coli* strains do not ferment sorbitol. Many studies to isolate *E. coli* O157:H7 have used sorbitol MacConkey agar. For example, Lupindu and friends [14], instead of focusing on every brown-colored, medium-sized round colony grown on MacConkey agar, they went for nonsorbitol fermenters in search for O157:H7. Sorbitol MacConkey agar was supplemented with antimicrobials cefexime and tellurite to inhibit growth of other bacteria such as *Aeromonas* and *Proteus* species and thus improving the recognition of nonsorbitol fermenting *E. coli*. The plates were inoculated with sample suspension and incubated at 37°C for 24 h. Nonsorbitol fermenting bacteria appeared colorless. NSF *E. coli* were confirmed by biochemical method. In this procedure, where one isolate was selected from each sample, the authors managed to recover 143 NSF *E. coli* isolates from the total of 1046 samples analyzed. The NSF *E. coli* isolates were further analyzed by molecular techniques, for example, PCR and DNA hybridization and serology to determine their virulence genes and pathotypes. Of 95 NSF *E. coli* isolates from cattle, 4 (4.2%) were *E. coli* O157:H7, carrying *vtx2c* genes.

Concentration of *E. coli* isolates can be achieved by molecular techniques where a specific part of the DNA is compared for different isolates. PFGE is one of the commonly used methods to bring together *E. coli* isolates with similar attribute prior to further analyses. Specific base pair sites of the DNA are cut by special enzymes, amplified, and electrophoresed by applying electric voltage in three directions periodically. It is suitable even for comparison of large DNA fragments up to 20 kb. PFGE can be reliably used as final analyses in the outbreak investigation in Ref. [12], but sequencing is becoming an adjunct to PFGE whereby isolates with identical PFGE bands are further subtyped by sequencing to give a more detailed discrimination in Ref. [15]. In outbreak situations, isolates are fingerprinted by PFGE, but detailed discrimination among isolates especially from different outbreak in different locations is obtained by sequencing. For example, Turabelidze and colleagues [16] sequenced pathogenic *E. coli* that was congregated by PFGE. It was reported that these isolates had identical PFGE band, but their differences were revealed by sequencing. Likewise, Trees et al. [17] sequenced 240 isolates related to outbreaks from different sources by PFGE fingerprinting. As a result, whole genome sequencing of 228 isolates showed

that they were Shiga toxin-producing *E. coli*, whereas other 12 isolates were non-Shiga toxin-producing diarrheagenic *E. coli*.

Moreover, *E. coli* isolates can be brought together by making use of common antigenic features they possess. Antibodies specific to the bacteria antigen are used to trap the bacteria in enrichment broth. Magnetic beads are coated with specific antibodies for specific bacteria antigen. When beads are applied to the culture broth, antigen will attract antibody resulting in bacteria-bead complex. The complexes are brought together by a magnetic field and concentrate at the bottom of the tube. After decantation, concentrated bacteria-bead complexes are inoculated on a solid media and incubated at 37°C for 24 h. The culture is then analyzed by other methods such as PCR or sequencing. Immunomagnetic separation (IMS) can be used to isolate different bacterial and fungal species. Different strains of Shiga toxin-producing *E. coli* can be isolated by this procedure. These include all Shiga toxin-producing *E. coli* with somatic antigen O157, O26, O45, O103, O111, O113, O121, and O145 [18].

Chromogenic media can also be used to concentrate bacteria possessing some enzymes whose action on sugars brings changes that are detected and depicted by indicating color change. *E. coli* are distinguished from other coliforms by the presence of β-glucuronidase activity on glucuronide. Examples of chromogenic media for coliform discrimination are Brilliance *E. coli* agar and Petrifilm Select *E. coli* count plate. Apart from differentiating the coliforms, these media can be used to sort out between β-glucuronidase positive and negative *E. coli* since there are a few *E. coli* strains that are β-glucuronidase negative, for example, *E. coli* O157:H7 [19]. Beta-glucuronidase positive isolates will appear purple on Brilliance *E. coli* agar or dark-green on Petrifilm Select *E. coli* count plate. The use of chromogenic media is usually followed by analyses by other techniques, for example, PCR, PFGE, or sequencing [20].

6. Storage of *E. coli* isolates

Preservation of bacteria aims at slowing the rate of harmful reactions in bacteria cultures so as to maintain viability and genetic attributes for future use. When imminent analyses require intact live cell, the storage method becomes very important. Different methods can be used to store pure *E. coli* and other bacteria isolates for future analyses [21]. Removal of water from the bacteria culture (drying) can be one option in preserving bacteria cells, while low temperature storage can also reduce the rate of chemical reaction in the cell culture and hence prolong bacteria viability. Drying of the bacteria cells may involve freeze and vacuum drying. In freeze drying, also called lyophilization or cryodesiccation, bacteria are suspended in a medium which maintain their viability through freezing, water removal, and storage. Principally, the bacteria in 15% glycerol suspension are frozen on dry ice or liquid nitrogen and subjected to high vacuum line that allows bacteria to dry through water sublimation. In vacuum drying, the bacteria are dried over calcium chloride in vacuum. Both freeze and vacuum-dried bacteria cultures are stored at 4°C for long time. Low temperature storage of bacteria involves keeping bacteria at low temperatures, ranging from 4 to −80°C. Freezing usually requires addition of glycerol or sugars as cryoprotectants. Deep freezing is the most common preservation method, which maintains both survival and similarity of bacteria population compared with other methods. The choice of the method of preservation depends on several factors, including the nature of bacteria, desired length of time of storage, analysis strategy, and study objectives.

Short period preservation, for example, for days or a week, bacteria can be stored under refrigeration temperatures. Pure bacteria culture is grown on agar slants or plates of nondifferential media and stored at 4°C. Screw-capped tubes are recommended when agar slants are used in bacteria preservation. Cultures on Petri dishes should be protected from contamination and rapid drying by sealing the plates with parafilm and stored inverted. Screw-capped tubes with hot sterile media are inclined at an angle to allow the media to solidify into a slant. A loopful of pure bacteria culture is inoculated onto the slant surface and incubated at 37°C for 24 h. The slant is then refrigerated for future use of bacteria.

Freezing is another method used to store bacteria whereby, the degree of coldness corresponds to length of storage period. The colder the storage temperature, the longer the culture will retain viable cells. Freezing temperatures of −20 to −40°C, which is achieved by most laboratory freezers, can be used to preserve bacteria for up to 1 year. Low temperature of −80°C can preserve bacteria for longer than 3 years, whereas cryofreezing at temperatures below −130°C, usually in liquid nitrogen, can preserve bacteria for more than 10 years.

Freezing may damage or kill bacteria cells due to resultant physical and chemical processes taking place. During freezing, water in the bacteria cell is converted to ice and solutes accumulate in the residual free water. Ice crystals formed can damage the cell membrane and the negative solute concentration can denature cell biomolecules. Cryoprotectants such as glycerol lower the freezing point of the bacteria suspension and thus prevent extracellular ice crystal formation and build-up of negative salt concentration. Besides, the lethal intracellular freezing is usually avoided by slow cooling or progressive freezing that allows sufficient water to leave the cell during freezing of extracellular fluid. A slow progressive freezing at a cooling rate of 1°C/min can be achieved by using a rate controlled freezer. Alternatively, similar results can be obtained by "snap freezing." Bacteria cells are snap-frozen by immersing the well-labeled 15% glycerol cell suspension containing cryotubes in dry ice or liquid nitrogen before storing them in freezer (−20 to −80°C) or in liquid nitrogen tank (−196°C) [22].

Bacteria cultures for freeze preservation can be prepared by inoculating a loopful of bacteria culture into nondifferential sterile broth such as nutrient broth followed by 37°C incubation for 24 h. This broth with pure bacteria culture is mixed with glycerol to make it 15–20% glycerol. Pure glycerol is a thick viscous liquid that needs dilution for practical handling. One-to-one dilution of pure glycerol with sterile normal saline is usually required, for example, 100 ml of glycerol is mixed with 100 ml of normal saline. As a result, for any required amount of pure glycerol, the diluted volume should be doubled. For example, if you want to store bacteria in 20% glycerol broth in a cryovial of 2 ml capacity, you need to put 600 μl of culture broth into a cryovial and add 400 μl of diluted glycerol. This 1 ml culture broth of 20% glycerol can be stored at −20, −80, or −196°C.

All *E. coli* strains can be revived by inoculation on blood agar, nutrient agar, or any nonselective media. A loopful of culture is inoculated onto the agar and incubated at 37°C for 18–24 h. Do not allow to thaw whenever frozen cultures intended for further storage are in use.

7. Characterization of *E. coli* isolates

Characterization includes detection of bacteria isolates from different sources and typing of bacteria isolates of same species. *E. coli* can be characterized by different methods, depending on what attribute is targeted. The methods are categorized as serology, molecular techniques, or cytopathic assays. Molecular characterization includes numerous techniques such as PCR, DNA hybridization, PFGE, restricted fragment length polymorphism (RFLP) and multilocus variable-number tandem repeat analysis (MLVA) to mention a few. These variable methods of bacteria typing have previously been summarized and compared in Ref. [19]. A combination of different methods can be used to complement each other especially when accurate diagnosis is required in a public health threat. A good example of combination of different characterization methods is the work reported by Sabat et al. [23], whereby isolates confirmed to possess somatic antigen O157 by agglutination test were further characterized by PCR subtyping of verotoxigenic (vtx) genes, O:H serotyping, Vero cell assay, sorbitol fermentation, β-glucuronidase activity, dot blot hybridization, and PFGE.

7.1. Serotyping

Presence of antigenic components that characterize a specific *E. coli* strain can be detected by using specific antibodies, for instance, presence of somatic antigen O, capsular antigen K, and flagella antigen H can be detected by agglutination tests and using specific antisera. The somatic and flagella antigens are tested against each specific antiserum, or they are tested against pools of antisera first and then tested against each of the specific antisera from the positive pools. The number of positive antisera is used in O and H antigen nomenclature, for example, *E. coli* O113:H21, O142:H34, and O157:H7. There are more than 180 O somatic antigens and more than 50 H-flagella antigens that are known and used as reference in *E. coli* serotyping. [24]. *E. coli* antigen serotyping has been described in detail by Ørskov and Ørskov [25].

7.2. Polymerase chain reaction (PCR)

Polymerase chain reaction is performed to characterize *E. coli* strains by targeting different virulence genes coding for different virulence factors. Common virulence factors for IPEC include verocytotoxin1, verocytotoxin 2, intimin, heat-stable enterotoxin, human variant, heat-stable enterotoxin, porcine variant, heat labile enterotoxin, and invasive plasmid antigen (**Table 2**). These virulence genes can be detected using multiplex DEC PCR kit as previously described in Ref. [26].

Virulence factor	**Gene target**	**Primer sequence (5′-)**	**Amplicon size (bp)**
Verocytotoxin 1	*vtx1*	GTTTGCAGTTGATGTCAGAGGGA	260
		CAACGAATGGCGATTTATCTGC	
Verocytotoxin 2	*vtx2*	GCCTGTCGCCAGTTATCTGACA	420
		GGAATGCAAATCAGTCGTCACTC	
Intimin	*Eae*	GGYCAGCGTTTTTTCCTTCCTG	377
		TCGTCACCARAGGAATCGGAG	
Heat-stable enterotoxin-human	*estA*-human	TTTCGCTCAGGATGCTAAACCAG	151

Virulence factor	Gene target	Primer sequence (5′-)	Amplicon size (bp)
Heat-stable enterotoxin-porcine	*estA*-porcine	CAGGATTACAACACAATTCACAGCAGTA CTTTCCCCTCTTTTAGTCAGTCAACTG	160
Heat-labile enterotoxin	*eltA*	CAGGATTACAACAAAGTTCACAGCAG AAACCGGCTTTGTCAGATATGATGA	479
Invasive plasmid antigen	*ipaH*	TGTGCTCAGATTCTGGGTCTCCT TTGACCGCCTTTCCGATACC	647
		ATCCGCATCACCGCTCAGAC	

Table 2.

Gene target, primer sequence, and amplicon size for common intestinal pathogenic *E. coli* virulence factors (Adapted from Persson et al. [26]).

EXPEC commonly carry virulence factor causing urinary tract or nervous tissue infection characterized by syndromes such as urosepsis, pyelonephritis, prostatitis, cystitis, and meningitis. More than 30 virulence factors carried by EXPEC have been reported in Refs. [27, 28]. These include *papA*, *papC*, *papEF*, *papG*, *papG* II (±III), *papG* III (±II), *papG* II + III, *sfa*, *focDE*, *sfaS*, *focG*, *afa*/*draBC*, *iha*, *bmaE*, *gafD*, *fimH*, *hlyD*, *cnf1*, *cdtB*, *fyuA*, *iutA*, *iroN*, *ireA*, *kpsM* II, K1 *kpsM*, K2 *kpsM*, *kpsMT* III, *rfc*, *cvaC*, *traT*, *iss*, *ibeA*, *ompT*, H7 *fliC*, *malX*, and *ibeA*. Commercial multiplex PCR kits are available for detection different virulence genes for EXPEC.

Verocytotoxin (*vtx*) genes form the most variable group of IPEC virulence factors that can further be characterized by PCR into *vtx1* and *vtx2*. Within *vtx1* and *vtx2* groups further subtyping can be done as previously described in Ref. [29]. As a result, 10 subtypes have been identified, three for *vtx1* (*vtx1a*, *vtx1c* and *vtx1d*) and seven for *vtx2* (*vtx2a*, *vtx2b*, *vtx2c*, *vtx2d*, *vtx2e*, *vtx2f* and *vtx2g*). This subtyping is important because the subtype differ in virulence and disease syndrome they cause. Moreover, these details are needed when comparison of isolates from different cases/outbreaks is desired.

Detection of virulence factors and genetic relatedness of *E. coli* isolates can also be assessed by DNA hybridization. This a phenomenon whereby a single strand of DNA anneals to a complementary single-stranded DNA fragment (probe) to form a hybrid. Since the probe is labeled, formation of a hybrid molecule is detected and hence showing presence of its complementary (target) nucleic acid strand. Apart from detection of conventional virulence genes, DNA hybridization can be used as a complementary to PCR to check for additional virulence factors [14, 30]. Analyses of additional virulence factors by hybridization can assist in differentiation of closely related isolates. For instance, EPEC pathotypes possess *eae* gene, and they can be differentiate into classical EPEC and A/EEC through DNA hybridization. Classical EPEC possesses *bfp* that codes for bundle-forming pili (BFP) [14, 31]. Different DNA probes can be used in hybridization such as *vtx1*, *vtx2*, *eae*, enterohaemolysin (*ehxA*), EPEC adherence factor

(*EAF*), bundle-forming pilus (*bfpA*), *saa*, *astA*, *and vtx2f*. The protocols for DNA hybridization have previously explained in Refs. [30, 32, 33].

7.3. DNA sequencing

This is the determination of precise order of bases in the nucleotides that make a specific segment of a DNA. Apart from characterization of genetic material for the purpose of identification of *E. coli* strain, DNA sequencing assist in comparison of genetic makeup from different sources, for example, in assessment of the association of different disease outbreak. Generally, sequencing use electrophoresis to separate pieces of DNA into bands. DNA molecules move through the gel when an electric current is applied and molecules are separated according to size, small molecules move faster. During sequencing, bases are tagged with fluorescence dyes, each base type producing a different color, for example, thymine = blue, cytosine = green, adenine = red, and guanine = yellow. Artificial modified bases are added to the DNA mixture. DNA molecules will undergo copying many times. When one of the modified bases is incorporated into the DNA molecule, elongation of the chain stops and all DNA pieces in that batch will have an ending with that particular modified base. The next batch of DNA copy will have a different artificial base at the end and so on. As a result, different DNA batches will end with different base T, A, G, and C, each with a specific color. So the base sequence in the assembled DNA material will be determined by a color pattern of the last (modified) base. The information is stored in computer memory and used for interpretation. This is a traditional Sanger sequencing. Besides, the fast advancing technology is taking the investigative life science from a few DNA fragments analysis into another level of whole genome sequencing. Next Generation Sequencing analyses the entire genome in a short time of single sequencing run. As a result, analysis and comparison of whole genome of isolates lead to correct diagnostic inference. Principally, next generation sequencing is similar to conventional Sanger method, but the former, through sequencing by synthesis, allows detection of single bases as they are incorporated into a growing DNA strand until the whole genome is read. Moreover, millions of reactions take place in parallel and many samples can be analyzed at once.

Sequencing is superior to other methods in characterization of genetic material. For example, whole genome sequencing can detect false positive and false negative clonal relationship of isolates from PFGE fingerprinting [34]. Regardless of the approach to the genome as a whole, the actual process of DNA sequencing is the same. Guidelines and protocols for sequencing are described in detail by a number of researchers in Refs. [35, 36], such that it is possible for many laboratories to manage the procedure.

7.4. Phenotypic characterization of *E. coli*

The genetic expression of *E. coli*, especially pathogenic *E. coli*, can be evaluated by applying the toxin extract from the bacteria to the monolayer Vero cell culture. Cytopathic effects to the cells will indicate virulence activities of the genes. Details of cytotoxic effect assay on Vero cell have been documented in Ref. [37]. Mouse inoculation can also be done to assess virulence of genes.

8. Common *E. coli* pathotypes

Intestinal pathogenic *E. coli* form a large proportion of pathogenic *E. coli*. They include VTEC, EPEC, ETEC, EAEC, DAEC, and EIEC.

Verocytotoxigenic *E. coli* (**VTEC**) produces verocytotoxins also known as Shiga toxins. The most common VTEC is O157:H7 strain. VTEC are characterized by possession of genes encoding for *vtx1* and *vtx2*, although they carry other virulence genes such as *eae* and *ehxA*. Animals are principal reservoirs of VTEC, and the main route of transmission is fecal-oral. In humans, especially children and elderly, VTEC cause abdominal cramps associated with diarrhea or dysentery. Complicated cases of VTEC infection may lead to HUS. VTEC can be isolated from different sources by different approaches, but the choice will depend on the objectives. Reliance on sugar fermentation ability, for example, sorbitol or presence of specific enzymes, for example, beta-glucuronidase, may lead to focus on specific fraction of the pathogen. On the other hand, targeting verocytotoxin-producing genes will give the overall burden of VTEC from a target source. In this scenario, the use of IMS technique may be recommended [28]. Characterization of isolates for VTEC detection may include immunological methods by using specific antibodies against target VTEC strain or PCR by targeting specific genes. VTEC isolates typing can be done by serology, using specific antisera, PFGE, DNA hybridization, and sequencing.

Enteropathogenic *E. coli* (**EPEC**) possess *eae* just as do some VTEC strains. As a result they cause attaching and effacing lesion and hence diarrhea. Classical EPEC differs from atypical EPEC (A/EEC) by possession of *bfpA* gene. However, atypical EPEC is a more prevalent cause of diarrhea [38]. Human EPEC infection follows fecal-oral route and isolation can be done from different sources such as water, food, animal, and environment. However, characterization emphasize should be put on distinguishing EPEC from VTEC by presence of *eae* gene and absence of vtx genes. Also, classical EPEC and atypical EPEC should be differentiated by assessing the presence of *bfpA* gene that encode for bundle-forming pili. These features can be determined by characterization procedures such as PCR and DNA hybridization [14]. PFGE typing can be applied to compare strains during outbreaks.

Enterotoxigenic *E. coli* (**ETEC**) are responsible for watery diarrhea in humans due to impaired sodium absorption and enhanced chloride secretion caused by enterotoxins. Fecal-oral contamination is responsible for transmission through food and water, and the syndrome is common to travellers and children. A simple procedure for detection of ETEC from stool has been described earlier in Ref. [38]. Heat-stable and heat-labile enterotoxins encoded by heat-stable enterotoxin (*estA*) and heat-labile enterotoxin (*eltA*) genes, respectively, are responsible. These genes can be easily detected by serological assays [39] and multiplex DEC PCR.

Enteroaggregative *E. coli* (**EAEC**) causes acute and persistent diarrhea in humans. This group has diverse strains differing in many aspects but have a common feature of forming a “stacked brick” pattern of adhesion to the human epithelial cell line HEp-2. This feature is used in HeLa cell adherence method to detect EAEC strains [40]. They often produce heat-stable toxin EAST1, Shigella enterotoxin (ShET1), and Haemolysin E, which cause host cell damage and induce inflammation leading to diarrhea especially in travellers, children, and immunocompromised patients. The EAEC strains are found in mixed infections whereby isolation by MacConkey ager, detection by conventional biochemical methods, and PCR and typing by PFGE are possible [41].

Diffusely adherent *E. coli* (DAEC) are responsible for acute diarrhea in humans. DAEC are characterized by the ability to adhere to Hep-2 cells in a diffuse fashion as confirmed by HeLa cells assays. Isolation is done conventionally and detection by PCR can be done by targeting Afa/Dr genes [42].

Enteroinvasive *E. coli* (EIEC) cause profuse diarrhea or dysentery in human through mechanical damage of host epithelial cell by using adhesin protein for binding and invading/entering intestinal cells. They do not produce toxin. EIEC resembles Shigella species biochemically and genetically. Most of them do not ferment lactose. Following conventional isolation methods, EIEC are detected by invasion plasmid antigens (*ipaH*) gene-targeted PCR [43]. The invasiveness of EIEC can be assessed by plaque formation on HeLa cell or guinea pig conjunctivitis assays.

Extra-intestinal pathogenic *E. coli* (EXPEC) cause a wide range of bacteraemia-associated disease syndromes. EXPEC have been isolated in patients with cystitis, pyelonephritis, or prostatitis [28]. Other syndromes associated with EXPEC include septic arthritis or pyomyositis, nontraumatic meningitis, or hematogenous osteomyelitis and pneumonia [44]. This group is comprised of UPEC, NMEC, and SEPEC [1]. Infection normally follows fecal-oral route. Samples to collect will depend on infected system; urine samples can be collected for urinary tract infection-related syndromes, such as cystitis, Pyelonephritis, or Prostatitis [28], whereas blood, joint fluid, psoas fluid, or sputum are target samples when nonurinary syndromes are concerned [44]. Isolation of *E. coli* for EXPEC detection can follow methods that have been mentioned previously for other pathotypes. Detection of EXPEC can be done by multiplex PCR targeting different genes some of which have been previously described and dot blot hybridization [1, 20, 27, 28]. Typing of isolates from different sources can be done by different procedures including PFGE [20].

9. The viable but nonculturable (VBNC) state

E. coli viability has been reported to decrease when the cells are exposed to direct sunlight because they enter a viable but nonculturable (VBNC) state, while retaining pathogenic ability [45]. Some factors that are directly or indirectly linked to sample collection, storage, or processing may contribute toward *E. coli* entering VBNC state. These include nutrient starvation, elevated or lowered osmotic concentration, oxygen concentration, exposure to heavy metals or food preservatives, direct sunlight, and incubation outside normal temperature range [46]. These factors may lead to false-negative outcomes because *E. coli* does not grow on standard laboratory media when they are under VBNC state. When some of VBNC inducing factors are difficult to avoid, then *E. coli* detection methods that do not rely on viable or live cells, for example, DNA-dependent methods such as PCR, can be a perfect option.

10. Conclusion

Dealing with a diverse group of bacteria like *E. coli* may present a challenge. Knowledge on basics of *E. coli* in terms of isolation and characterization may help in planning, setting objectives, and execution of *E. coli*-related research. One should bear in mind that choice of one isolation or characterization approach may lead to a different output compared to another approach.

The current procedures for *E. coli* isolation and characterization take at least 72 h and sometimes even more time. The need to work on viable bacteria cells may be contributing much to this lengthy procedure. Working on the genetic material right from the sample could help to shorten the time spent from isolation of *E. coli* from sample to outcome. This should be the direction of future research.