**GLOBAL ACADEMIC RESEARCH INSTITUTE**

COLOMBO, SRI LANKA


**GARI International Journal of Multidisciplinary Research**

ISSN 2659-2193

**Volume: 06 | Issue: 04**

On 31st December 2020

http://www.research.lk

Author: Michael Harvie, Uthpala Amarasekara

BMS, School of Science, Sri Lanka

GARI Publisher | Public Health | Volume: 06 | Issue: 04

Article ID: IN/GARI/ICAS/2020/109 | Pages: 60-82 (23)

ISSN 2659-2193 | Edit: GARI Editorial Team

Received: 07.10.2020 | Publish: 31.12.2020

# IDENTIFICATION OF THE PRESENCE OF ANTIBIOTIC GENES IN ESCHERICHIA COLI IN DRINKING WATER SAMPLES OBTAINED FROM THE MALABE REGION

Michael Harvie, Uthpala Amarasekara

*BMS, Sri Lanka*

## *ABSTRACT*

Antibiotic resistance is a threat that has manifested into a global crisis, causing several fatalities yearly. In developing countries, the prevalence of antibiotic resistance is rapidly increasing due to sub-standard disinfection and clearance protocols of hospital and household sewage and waste matter, and inadequate surveillance. Surface and ground waters are hot-spots for the dissemination of antibiotic resistance via horizontal and vertical gene transfer leading to the development of multidrug resistant bacteria capable of causing fatal infections. Escherichia coli is a known vector of antibiotic resistance and a commonly studied waterborne pathogen. For the isolation and differentiation of E. coli from other coliforms, initial membrane filtration and chromogenic culturing was carried to allow a non-specific size-based separation followed by colony colour based selection of potential E. coli isolates. The Simmon's citrate agar test was performed which showed negative results for 9 samples, followed by the indole test which showed positive results for 10 samples. Further, molecular analysis was performed wherein it was found that 10 samples were positive for the 16SrRNA gene, all samples possessed the uidA gene and 9 isolates carried the LacY gene. Disc-diffusion antibiotic sensitivity testing on the 9 isolates revealed samples 2, 5, 6, and 11 were resistant to tetracycline, however sulfonamide sensitivity was observed among all samples. Molecular analysis of tetA and tetC genes showed that sample 6, collected from a lake showed positive results for tetA, whereas sample 2, collected from a tube well, and samples 5 and 11 from fishing site possessed the tetC gene.

Keywords: Antibiotic resistance, Escherichia Coli, Tetracycline resistance, Sulfonamide resistance.

## *INTRODUCTION*

Antibiotic resistance

The discovery and development of antibacterial agents in the early 1900s was a breakthrough, which provided an effective treatment strategy and dramatically reduced the mortality rates due to bacterial infections (Davies and Davies, 2010). In modern times however, the term 'antibiotic resistance' which refers to the ineffectiveness of antibiotics to treat bacterial infections, has become increasingly common (Perry, 2019). Socioeconomic factors such as lack of education on the topic, poor surveillance, overuse and misuse in clinical and agricultural settings exacerbate the development and rapid spread of AR (Chokshi et al., 2019; Jayaweerasingham et al., 2019; Berglund, 2015). Antibiotics act as selection pressures on bacteria and organisms that develop or acquire resistance are selected. The organisms can then disseminate ARGs vertically and horizontally leading to several strains and species of AR and MDR organisms.

Infections by such organisms account for up to 700,000 fatalities annually (WHO, 2020; Aslam et al., 2018; Collington et al., 2018; Toner et al., 2015). Water bodies can be contaminated with ARB and ARGs from agricultural, household or hospital waste/sewage. Once consumed, ARB in the water can cause pathogenic effects and the ARGs can be further disseminated (Fig.1) (Watts et al., 2017).

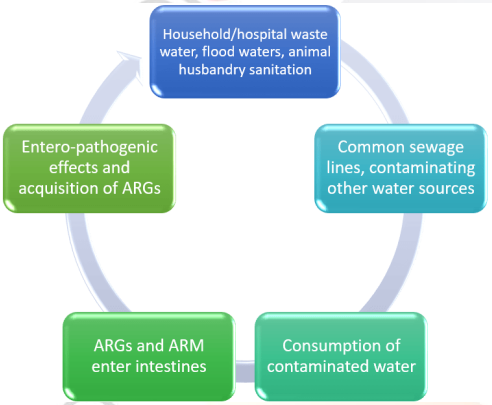


*Figure 1:* The dissemination mechanism of ARGs. ARGs contaminate water bodies and remain in surface or ground-water. Pathogenic effects and faecal contamination of water via sewage can occur following consumption (Adapted from: Sanganyado and Gwenzi, 2019).

**Tetracycline resistance**

Tetracyclines exhibit bacteriostatic effects on a wide range of gram negative and gram positive organisms by binding to the 30s domain of 70s bacterial ribosomes, inhibiting the binding of aminoacylated-tRNA molecules to the A-site. This inhibits protein synthesis, therefore arresting growth and metabolic processes of the bacterium (Nicolaou and Rigol, 2017; Nguyen et al., 2014).

A plethora of genes contribute to tetracycline resistance via mechanisms such as; efflux, ribosomal protection and enzymatic degradation. Novel research has also revealed a TetU resistance gene, however, the mechanism of action remains unclear (Grossman, 2016; Roberts and Schwarz, 2017).

Tetracycline efflux mechanisms are mediated by the "Major Facilitator Superfamily" which are transmembrane proteins that actively transport tetracycline out of the bacterial cell, facilitated by the exchange of a proton into the cell (Fig.2). The tetA and tetB genes are more commonly found in the gram negative bacteria, whereas tetK and tetL are more prevalent in the gram positive bacterium (Roberts, 2020).

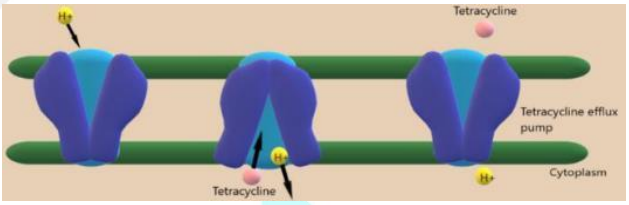


*Figure 2*: Mechanism of action of the tetracycline efflux pump. Influx of a proton can induce a conformational change to the channel, causing tetracycline molecules to be pumped out of the cell (Adapted from: Nguyen et al., 2014).

**Sulfonamide resistance**

The bacteriostatic properties of the sulfonamides were first noted in the 1930s and its derivatives became widely used to treat bacterial pneumonia and UTIs by the 1940s. The enzyme DHPS catalyses the reaction between dihydropteroate diphosphate and PABA into tetrahydrofolate. Sulfonamides exhibit a similar structure to PABA (Fig.4(A)) allowing it to competitively inhibit DHPS, which inhibits the formation of tetrahydrofolate (Fig.4(B)), consequently arresting DNA synthesis (Yousef et al., 2018; Capasso and Supuran, 2020).

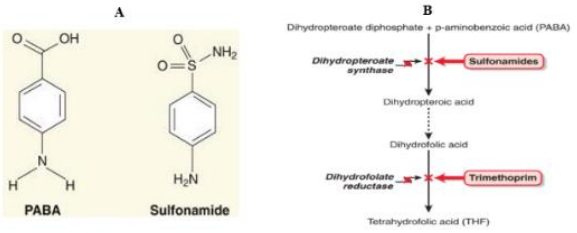


*Figure 3: Sulfanamide structure and mechanism of action.* A) The similarity between sulfonamides and PABA; B) The targets effects of sulfonamides (Bardal *et al.*, 2011).

Research shows that bacteria acquire resistance to sulfonamides by either spontaneous mutation in the folP gene which codes for DHPS, or by acquisition of the plasmid genes; sul1, sul2, sul3, and the novel sul4 variant which code for structurally diverse forms of DHPS that have a low affinity for sulfonamides (Razavi et al. 2017; Jiang et al., 2019). Furthermore, the flavin-dependent monooxygenase SulX enzyme, coded by the sulX, gene causes resistance to sulfonamides by enzymatic degradation (Kim et al., 2019).

**Escherechia Coli**

Escherechia Coli is a bacterium of great interest in the modern world and several commensal, pathogenic and environmental strains exist. Though the organism exists within the intestinal flora, several pathotypes exist such as; Enterotoxic, Shigatoxic, Enteroaggravative, Enteroainvasive, diffusely adherent, Enteropathogenic, Enterohemorrhagic, Enteroaggravative shigatoxic (DEC) and the uropathogenic and neonatal meningitis E. Coli (ExpEC) strains which cause disease. E. Coli is also a common contaminant of waterbodies and is a known vector of ARG dissemination. (Barbau-Piednoir et al., 2018; Miotto et al., 2018; Larson et al., 2019; Pleguezuelos-Manzano et al., 2019).

Distinguishing features of Escherechia Coli

Though there are several variations (genetic and otherwise) within the species, there are multitudinous similarities that allow for the unique identification and analysis of E. Coli. This particular bascillus possesses a thin peptidoglycan cell wall, therefore, morphological analysis of Gram stained E. Coli would result in the visualization of pink, rod shaped bacteria. Regulated by the tnaL (leader peptide), tnaA (tryptophanase), and tnaB (tryptophan permease) genes of the tna operon, E. Coli utilize and ferment tryptophan for metabolic processes, which is an attribute that answers the indole test (Han et al., 2011; Orozco-Gómez et al., 2019). E. Coli also metabolize glucuronide using β-glucoronidase, coded by the uidA gene, which allows differential identification when cultured on glucuronide rich media (Sivri et al., 2016). Contrastingly, E.Coli is unable to metabolize citrate and therefore does not thrive on citrate rich media (Leon et al., 2018).

**Significance of the study**

Studies show that the presence of antibiotic resistant strains of E. Coli and other organisms are rising within Sri Lanka (Kumar et al., 2020; Liyanage and Manage, 2019; Priyadharshana et al., 2019). This study aims to analyse the conditions and contributors of antibiotic resistance in a selected population. Over the last decade a population increase of over 30% has been observed in the urbanized city of Malabe, which has been correlated with an increase in pollution and depletion in ground water quality (Fernando and Rathnayake, 2018). This archeo-ecological wet-zone receives a significant amount of groundwater from the Kelani river and villagers of the outskirts rely on the water from streams branching off the river and wells for consumption, fishing, sanitation and bathing purposes. Studies show that the Kelani river harbours several coliform contaminants of up to 500cells/mL, more

specifically, with E. Coli prevalence of up to 57CFUs/mL. Several strains isolated show multidrug resistance and a high prevalence of tetracycline and sulfonamide genes, among others, have been identified (Kumar et al., 2020; Kumar et al., 2020; Surasinghe et al., 2019). By analysing the presence of common antibiotic resistant genes in E. Coli present in these highly used water bodies, the prevalence of antibiotic resistance can be roughly determined within the region. Furthermore, the results obtained can potentially be coupled with other similar studies in order to assess the status of antibiotic resistance within the country and to educate the high risk populations about the dangers, and preventive measures of antibiotic resistance.

## *METHODOLOGY*

**Measurement of physicochemical parameters**

The pH and temperature of the 10 water samples were measured using an electronic probe and chloride strips were used to estimate the chloride concentration.

**Membrane filtration**

25.00mL of chromogenic agar was poured into 24 petri plates and allowed to solidify. A 0.45µm pore membrane filter was placed in a Buchner funnel and 100mL of the first water sample was filtered. The filter was then placed on the corresponding chromogenic agar and the plate was parafilmed. This was repeated for the same sample to create a duplicate and the process was repeated for the remaining samples, positive (ATCC25922 E. coli) and negative (autoclaved distilled water) controls. All plates were inverted and incubated at 37oC for 24hours and CFUs were counted the next day.

**Colony isolation**

Into 12 falcon tubes, 5.00mL of LB broth was poured and allowed to cool. Colonies of interest from each chromogenic culture were selected with a sterilized inoculation loop and transferred into the corresponding falcon tube and the tubes were incubated at 37℃ for 24hours.

The next day, the falcon tubes were observed for turbidity. Into 11 petri plates, 25.00mL of nutrient agar was poured and allowed to cool. Quadrant streaking of each inoculum was carried out onto the corresponding plate. The plates were sealed, inverted and incubated at 37℃ for 24hours.

Into 12 labelled falcon tubes, 5.00mL of LB broth was poured and allowed to cool. Isolated colonies from each plate were selected and inoculated into the corresponding tube, which were incubated at 37℃ for 24hours and then refrigerated.

Biochemical (Citrate and Indole test) and morphological (Gram staining) analysis

Into 14 autoclaved test tubes, 5.00mL of Simmon's citrate agar was poured and allowed to solidify at a slant. A sample of each sub-culture, the ATCC25923 S.Aureus and ATCC25922 E.Coli mother-cultures, was swabbed onto the corresponding citrate agar. The tubes were sealed with cotton and foil and incubated at 37oC for 24hours and visualized the next day.

Consecutively, into 13 autoclaved test tubes, 5.00mL of tryptophan broth was poured. Each tube was inoculated with the corresponding sub-culture and controls. The tubes were covered with foil and incubated at 37oC for 24hours. The next day, 0.5mL of Kovack's reagent was added to each tube and the interface was observed.

Grams-staining was carried out for S1-S11 and 40x and 100x microscopic observations were performed.

Genomic DNA Extraction and visualization

An overnight sub-culture of all samples and positive control was prepared. Genomic DNA was extracted from all samples using the Promega kit. 5.00µL of each extracted sample was mixed with 2.00µL of loading buffer and subjected to 0.8% agarose gel electrophoresis at 60V for 25 minutes, and then visualized using UV trans-illumination. The extracted samples were stored at 4oC.

**Polymerase Chain Reaction (16SrRNA, uidA and LacY) and product visualization**

Primers for 16SrRNA (F: 5'-GTTGTAAAGCACTTTGAGTGGTGAGGAAGG-3'; R: 5'-GCCTCAAGGGCACAACCTCCAAG-3') (Gao et al., 2012), uidA (UALF: 5'-TGGTAATTACCGACGAAAACGG-3'; UAR: 5'-ACGCGTGGTTACAGTCTTGCG-3') (Molina, et al., 2015) and LacY (F: 5'-ACCAGACCCAGCACCAGATAAG-3'; R: 5'-TTCTGCTTCTTTAAGCAACTGGC-3') (Lobersli et al., 2016) were diluted into 2.5µM solutions.

Separate PCR mixtures were prepared for and 16SrRNA, uidA and LacY. Each mixture consisted of 5.00µl of 5x PCR buffer, 0.625µl of 10.00mM dNTPs, 2.50µl of 2.5µM forward and reverse primers, and 1.50µl of 25.00µM MgCl2. This was followed by adding 1.50µL of the corresponding extracted DNA, and 1.50µL of distilled water for the blank control.

The PCR mixtures for the identification of the 16SrRNA gene were placed in the thermal cycler and run at 94°C for 2 minutes (initial denaturation), 35 repetitions of 94°C for 30 seconds (denaturation), 55°C for 45 seconds (annealing), 72°C for 90 seconds (extension), and 72°C for 10 minutes (final extension) (adapted from He et al., 2007). The PCR mixtures for the identification of the uidA gene were placed in the thermal cycler and run at 95°C for 3 minutes (initial denaturation), 35 repetitions of 95°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), 72°C for 60 seconds (extension), and 72°C for 10 minutes (final extension) (adapted from Fatemeh et al., 2014). For the identification of the LacY gene, the mixtures were placed in the thermal cycler and run at 94°C for 3 minutes (initial denaturation), 35 repetitions of 94°C for 60 seconds (denaturation), 58°C for 30 seconds (annealing), 72°C for 60 seconds (extension), and 72°C for 5 minutes (final extension) (adapted from Lobersli et al., 2016).

Samples were then subjected to 2.0% agarose gel electrophoresis at 50V for 20 minutes and 45V for 40 minutes and visualized using UV trans-illumination.

**Antibiotic Disk Diffusion**

Selected samples and positive control cultures were inoculated in fresh LB broth and incubated at 37°C. Following 24hours, 20 petri plates were labelled, segmented into three divisions; T-tetracycline, S-Sulfonamide, N-Negative, and filled with 25.00mL of MHA. Standard solutions of each overnight culture were prepared by inoculating the culture drop-wise into 5.00mL of autoclaved, distilled water while turbidity was compared with a 0.5McFarland solution against a Wickerham card. Following the Kirby-Bauer disc diffusion protocol each standard culture was swabbed on to the corresponding plate, and 30µg tetracycline, 300µg sulfonamide and autoclaved distilled water discs were placed appropriately. The plates were incubated at 37°C for 24hours. The following day, the zones of inhibition were measured and compared with CLSI standards (Table 6).

**Table 1:CLSI standards for zones of inhibition**

| Antibiotic | Tetracycline 30µg (mm) | Sulfonamide 300µg (mm) |
|---|---|---|
| **Sensitive** | ≥15 | ≥16 |
| **Intermediate** | 12 -14 | 11-15 |
| **Resistant** | ≤11 | ≤10 |

**PCR (tetA and tetC) and product visualization**

Primers for tetA (F: 5'-GCTACATCCTGCTTGCCTTC-3'; R: 5'-CATAGATCGCCGTGAAGAGG-3') and tetC (F: 5'-TGCGTTGATGCAATTTCTATGC-3'; R: 5'-GGAATGGTGCATGCAAGGAG-3') (Chen et al., 2013) were diluted into 2.5µM solutions.

Separate PCR mixtures were prepared for and tetA and tetC gene identification using the same volumes and concentrations previously stated.

The PCR mixtures for the identification of the tetA gene were placed in the thermal cycler and run at 95°C for 5 minutes (initial denaturation), 40 repetitions of 95°C for 20 seconds (denaturation), 60°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and 72°C for 10 minutes (final extension) (Chen et al., 2013). The PCR mixtures for the identification of the tetC gene were placed in the thermal cycler and run at 95°C for 5 minutes (initial denaturation), 40 repetitions of 95°C for 20 seconds (denaturation), 64°C for 40 seconds (annealing), 72°C for 30 seconds (extension), and 72°C for 10 minutes (final extension) (Chen et al., 2013).

Samples were then subjected to 2.0% agarose gel electrophoresis at 50V for 20 minutes, then 45V for 40 minutes and visualized using UV trans-illumination.

## *RESULTS*

Sample collection data and physiochemical parameters of the water samples

Table 2: Sources and physiochemical parameters of each sample

| Location | Source | pH | Avg. pH | Temperature (ºC) | Avg. temperature (ºC) | Chlorine concentration (ppm) |
|---|---|---|---|---|---|---|
| 1 | Tap water | 6.95 | | 28.00 | | 5 |
| 2 | Filtered water | 7.02 | | 28.00 | | 5 |
| 3 | Stream | 7.03 | | 28.00 | | <5 |
| 4 | Fishing site | 6.82 | 6.984 | 29.00 | 28.30 | <5 |
| 5 | Tube well | 7.05 | 6.984 | 28.00 | 28.30 | <5 |
| 6 | Lake | 7.02 | 6.984 | 29.00 | 28.30 | <5 |
| 7 | Stream | 6.92 | | 28.00 | | <5 |
| 8 | Lake | 6.89 | | 28.00 | | <5 |
| 9 | Well (unprotected) | 7.02 | | 28.00 | | <5 |
| 10 | Well (protected) | 7.12 | | 29.00 | | <5 |

**Membrane filtration and chromogenic culture**

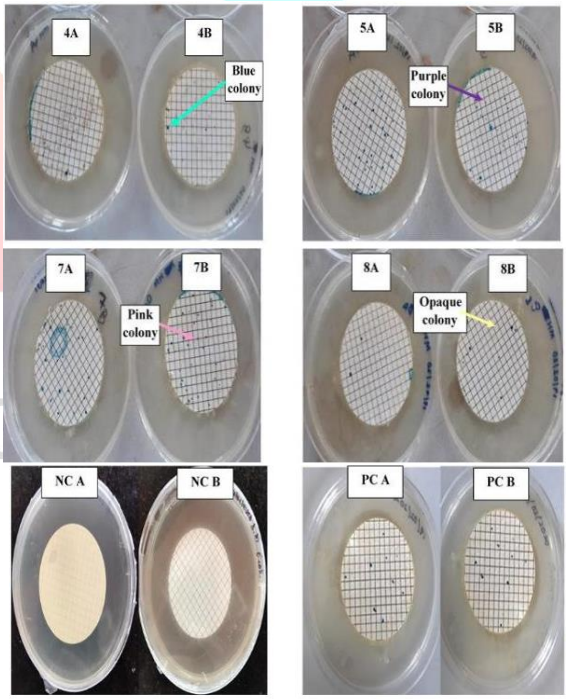


*Figure 4: Results obtained from membrane filtration and culture on chromogenic agar.* Samples from locations 1-10 and negative (Autoclaved distilled water) and positive (ATCC25922 *E.Coli*) controls in duplicates.

*Table 3: Colonies observed on chromogenic agar* (X-Colonies absent, ✓-Colonies present with exact count within brackets, highlighted-selected for sub-culturing).

| Sample location | Purple | Pink | Opaque | Blue-green |
|---|---|---|---|---|
| 1A | X | X | ✓ | X |
| 1B | X | X | ✓ | X |
| 2A | X | X | X | X |
| 2B | X | X | X | X |
| 3A | X | X | ✓ | ✓(7) |
| 3B | X | X | ✓ | ✓(12) |
| 4A | X | X | ✓ | ✓(8) |
| 4B | X | ✓(2) | ✓ | ✓(9) |
| 5A | ✓(6) | X | ✓ | ✓(30) |
| 5B | ✓(4) | X | ✓ | ✓(6) |
| 6A | X | X | ✓ | ✓(9) |
| 6B | X | X | ✓ | ✓(10) |
| 7A | X | ✓(1) | ✓ | ✓(40) |
| 7B | X | X | ✓ | ✓(30) |
| 8A | X | ✓(1) | ✓ | ✓(3) |
| 8B | X | ✓(3) | ✓ | ✓(10) |
| 9A | X | X | ✓ | X |
| 9B | X | X | ✓ | X |
| 10A | X | X | ✓ | X |
| 10B | X | X | ✓ | X |

**Table 4: CFUs of blue green colonies**

| Sample | CFUs |
|---|---|
| **3A** | 0.07 |
| **3B** | 0.12 |
| **4A** | 0.08 |
| **4B** | 0.09 |
| **5A** | 0.3 |
| **5B** | 0.06 |
| **6A** | 0.09 |
| **6B** | 0.1 |
| **7A** | 0.4 |
| **7B** | 0.3 |
| **8A** | 0.03 |
| **8B** | 0.1 |

**Biochemical and morphological analysis**

Simmon's Citrate test


*Figure 5: Simmon's citrate test observations.* Green indicates a negative result and blue indicates a positive result.

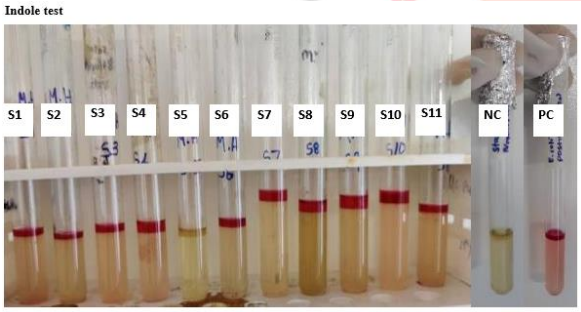


*Figure 6: Indole test observations.* Cherry red ring at interface indicates a positive result and yellow ring at interface indicates a negative result.

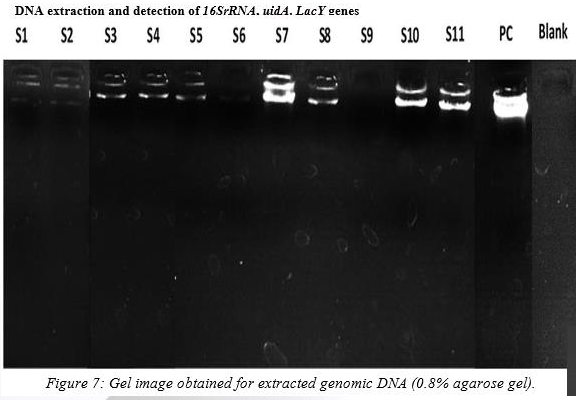


*Figure 7: Gel image obtained for extracted genomic DNA (0.8% agarose gel).*

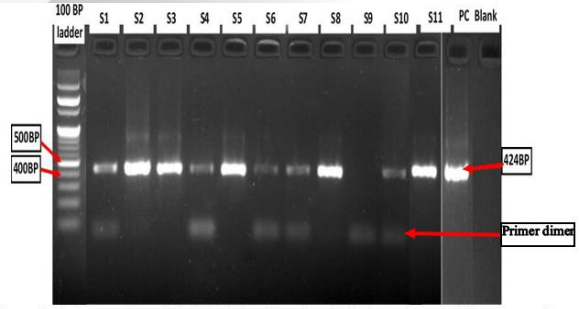


*Figure 8: Gel image obtained for 16SrRNA PCR product (424bp) visualization (2% agarose gel).*

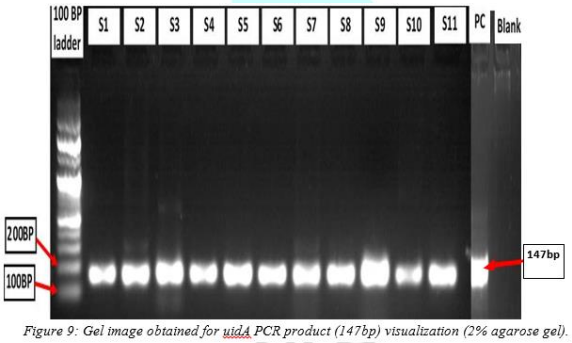


*Figure 9: Gel image obtained for uidA PCR product (147bp) visualization (2% agarose gel).*

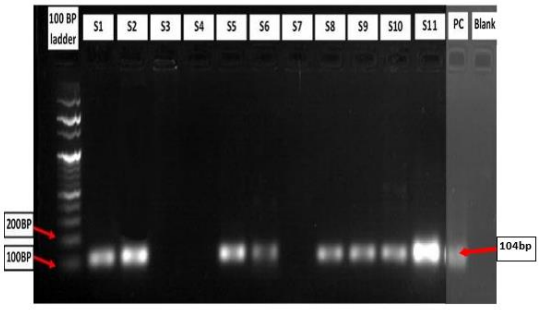


*Figure 10: Gel image obtained for LacY PCR product (104bp) visualization (2% agarose gel).*

Table 5: Summary of results obtained for biochemical and morphological testing

| Sample | Selected colony | Citrate utilization | Indole production | Gram staining identification |
|---|---|---|---|---|
| S1 | 5A Blue | Negative | Positive | Gram negative rods |
| S2 | 5B Blue | Negative | Positive | Gram negative rods |
| S3 | 5B Purple | Negative | Positive | Gram positive cocci |
| S4 | 3B Blue | Negative | Positive | Gram negative rods and cocci |
| S5 | 4B Pink | Positive | Negative | Gram negative rods and cocci |
| S6 | 8B Opaque | Negative | Positive | Gram negative rods |
| S7 | 7B Blue | Negative | Positive | Gram negative rods |
| S8 | 6A Blue | Positive | Positive | Gram negative rods |
| S9 | 8B Blue | Positive | Positive | Gram negative rods |
| S10 | 7A Pink | Negative | Positive | Gram negative rods |
| S11 | 4B Blue | Negative | Positive | Gram negative rods |
| E. Coli ATCC25922 | Mother culture | Negative | Positive | Gram negative rods |
| S. Aureus ATCC25923 | Mother culture | Positive | Negative | Gram positive cocci |

*Table 6:Summary of molecular identification results obtained for all samples* (X-no band observed, ✓-Bands observed and intensity elaborated within brackets where necessary, highlighted samples were selected for ABST).

| Sample | Genomic DNA (Promega) | *16SrRNA* | *uidA* | *LacY* |
|---|---|---|---|---|
| S1 | ✓ | ✓ | ✓ | ✓ |
| S2 | ✓ | ✓ | ✓ | ✓ |
| S3 | ✓ | ✓ | ✓ | X |
| S4 | ✓ | ✓ | ✓ | X |

| | | | | |
|---|---|---|---|---|
| S5 | ✓ | ✓ | ✓ | ✓ |
| S6 | ✓(faint) | ✓ | ✓ | ✓ |
| S7 | ✓ | ✓ | ✓ | X |
| S8 | ✓ | ✓ | ✓ | ✓ |
| S9 | ✓(faint) | X | ✓ | ✓ |
| S10 | ✓ | ✓ | ✓ | ✓ |
| S11 | ✓ | ✓ | ✓ | ✓ |

ABST observations following overnight incubation

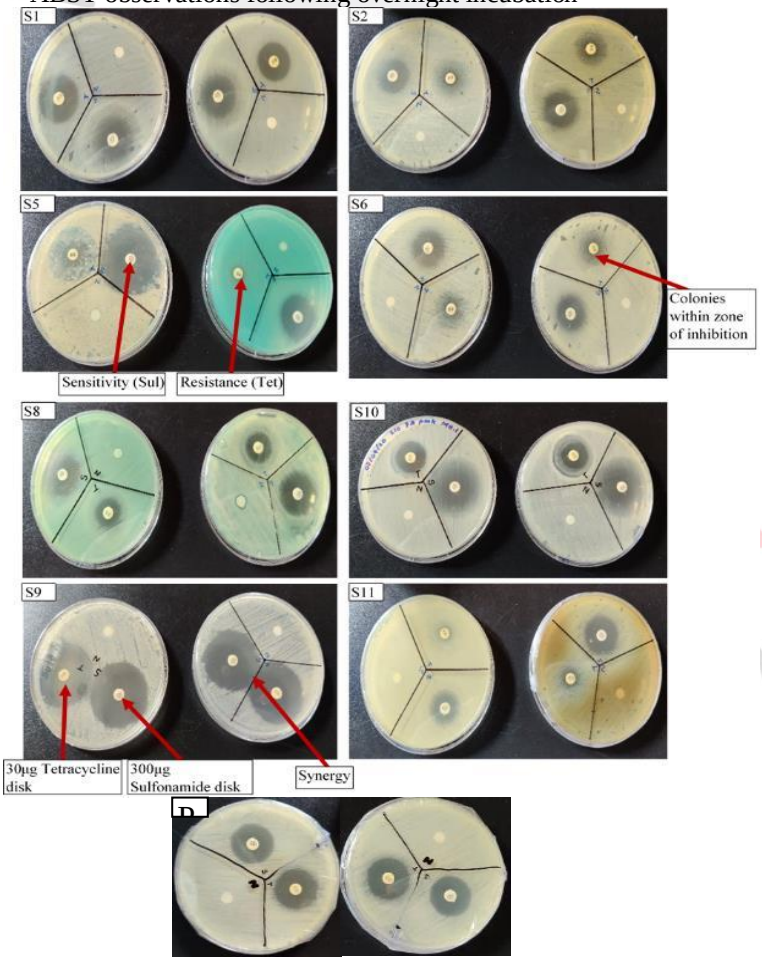


*Figure 11: Observations of ABST following*

Table 7: Results of the ABST in accordance with CLSI guidelines (highlighted samples were selected for downstream analysis).

| Sample | Tetracycline 30µg | | Sulfonamide 300µg | |
|---|---|---|---|---|
| | Average (mm) | Interpretation | Average (mm) | Interpretation |
| S1 | 22.00±2.83 | Sensitive | 18.00±0.00 | Sensitive |
| S2 | 9.25±0.35 | Resistant | 19.00±0.00 | Sensitive |
| S5 | 9.00±0.71 | Resistant | 25.25±3.89 | Sensitive |
| S6 | 9.50±0.71 | Resistant | 18.25±1.06 | Sensitive |
| S8 | 17.50±0.00 | Sensitive | 20.00±1.41 | Sensitive |
| S9 | 35.75±1.77 | Sensitive | 36.50±0.71 | Sensitive |
| S10 | 19.25±1.06 | Sensitive | 30.00±0.00 | Sensitive |
| S11 | 8.00±0.00 | Resistant | 20.25±1.06 | Sensitive |
| PC | 22.75±0.35 | Sensitive | 22.50±0.00 | Sensitive |

**Molecular detection of antibiotic resistance genes**

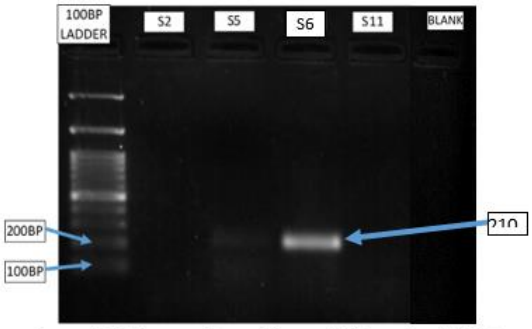


*Figure 12: Gel image obtained for tetA PCR product (210bp)*

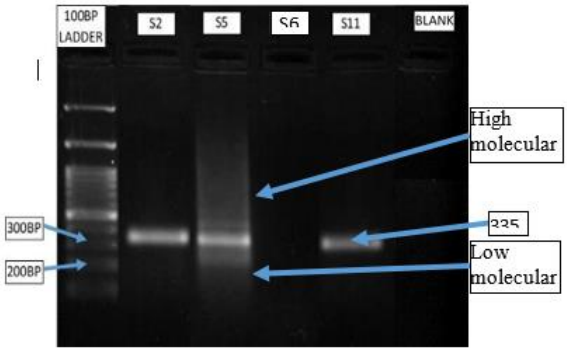


*Figure 13: Gel image obtained for tetC PCR product (335bp)*

Table 8:Summary of the identification of tetA and tetC genes

| Sample | TetA | TetC |
|---|---|---|
| S2 | X | ✓ |
| S5 | X | ✓ |
| S6 | ✓ | X |
| S11 | X | ✓ |

## *DISCUSSION*

Membrane filtration is an effective technique to separate contaminants from water as large volumes can filtered, and molecules and microorganisms larger than 0.45µm remain in the filtrate (Madeson, 2014; Hajj and Newman, 2015). Canonically, the HiMedia E. Coli chromogenic media is selective for E. Coli, which is ideal, since the filtrate would consist of various organisms (if any) due to its non-specific size based isolation. The chromogenic agent, X-Glucuronide, gives a bluish-green

appearance to colonies when the bond between the chromogen and glucuronide is cleaved by β-Glucuronidase, the product of the uidA gene. This gene is known to be approximately 97% specific among E. Coli, however, Salmonella, Shigella and Klebsiella spp. have also aquired this gene, allowing such colonies to appear bluish-green (HiMedia Labs, 2018; Little et al., 2017). Further, shigatoxic E. Coli (O157:H7) does not express the uidA gene due to evolutionary frameshift mutations, causing such colonies to have an off-white/opaque appearance (Monday, et al., 2001; Ogura et al., 2018). Moreover, the chromogenic compound found in the X-Glucuronide is an indolyl derivative, and the presence of the 5-bromo-6-chloro-indoxyl isoform can give rise to magenta/purple coloured colonies when the bond is cleaved. This therefore can possibly account for the presence of various coloured colonies observed on the chromogenic agars observed (Perry and Freydiere, 2007). Further possibilities relating to the presence of various coloured colonies is that certain species of bacteria express pigmentation when cultured. These include common gram negative contaminants of water such as Chromobacterium violaceum which produces purple 'violacin', and astaxnthin producing organisms which are pink (Kumar et al., 2015; Usman et al., 2017).

Due to such limitations, colony colour based identification of E. Coli isolates is inconclusive, therefore, testing biochemical parameters of the samples provides further insight into the identities of each colony. Testing the citrate utilization capability relies on the principle of citrate metabolism occurring simultaneously with ammonia production. Ammonia increases the pH of the medium which can result in bromophenol blue changing colour from green to blue, which is observed in the Simmon's citrate test (HiMedia Labs, 2018). However, organisms such as E. Coli and Shigella spp. are incapable of citrate utilization, therefore, the agar will remain green. This test requires highly regulated conditions as slight alterations of pH change the colour of the agar. Since the isolated colonies were sub-cultured in LB broth and stored for days, accumulation of waste metabolites in the culture can create an alkalinic pH, therefore, when swabbed on the agar, this can induce a false positive result (Stancik et al., 2002; Sezonov et al., 2007; Sánchez-Clemente et al., 2018). Further, the E. Coli K-12 strains possess citT citrate/succinate-antitransporter genes which can be expressed under anaerobic conditions to utilize citrate (Finn et al., 2017). Furthermore, citrate utilization under aerobic conditions can be achieved in some strains due to point mutations in the rnk promoters and the dctA genes which induce the expression of citrate transporters. Such strains can give rise to positive results as seen in samples S5, S8 and S9 (Hofwegen et al., 2016).

The indole test can differentiate coliforms based on the ability of the organism to catabolize tryptophan into indole, which is mediated by the tnaA and tnaB genes of the tna operon which code for tryptophanase and tryptophan permease, respectively. The Kovack's reagent can react with indole at the interface, giving a cherry-red ring which can indicate the presence of E. Coli which is capable of tryptophan utilization (HiMedia Labs, 2018). Some strains of E. Coli may have non-functional or mutated tna operons which are incapable of tryptophan utilization. Due to lack of tryptophan utilization, this can give a negative result upon addition of Kovack's reagent as S5 shows (Li and Young, 2015; Han et al., 2011). Serotypes of Shigella have variable indole test results where some strains are positive for indole production, whereas others are incapable due to mutational insertions into the tna operon, leading to inactive translational products (Rezwan et al., 2004).

Morphologically, E. Coli appear as gram negative bacilli (Percival and Williams, 2015). The process of Gram staining involves an initial heat fixing step which is prone to errors, as high heat is capable of inducing morphological variances potentially causing bacilli to appear as cocci or diplococci. Further, Gram staining is a test of low specificity and has approximately a 40% chance of giving false Gram positive results. These factors contribute to some variances seen in the results where cocci and Gram positive organisms were observed (S3, S4, S5) (Samuel and Pelbani, 2017; Samuel et al., 2017).

The differentiation between Shigella and E. coli is challenging as they express similar morphological and biochemical profiles, therefore, the use of molecular detection techniques in amalgamation with biochemical test results provides a distinctive confirmation of the presence of isolated E. Coli. Identification of the 16SrRNA gene is an effective determinant of the presence of bacteria, which can confirm that no fungal contaminants from the initial chromogenic plate were sub-cultured. This gene is conserved in all bacterial species, however, hypervariable regions within the gene and DNA recombination can show species based differentiation (Wang et al., 2015). Since variations are common within the 16SrRNA gene, the absence of an apparent 424bp 16SrRNA gene amplicon band following PCR (as seen in S9) can result primer-target mismatches due to mutations in the primer target region. Further, this can result from a low amplicon number due to insufficient cycle numbers due to a low concentration of template DNA (Witzke et al., 2020). Varying copies of the 16SrRNA gene may also exist within the organism, therefore, the primer target may be varied or absent, thus amplification may not occur (Winand et al., 2020). The shigella and E. Coli strains express less than 1% 16SrRNA sequence variance, therefore it is not possible to differentiate the species without further genetic analysis (Edwards et al., 2012; Jenkins et al., 2012). As aforementioned, the uidA gene is highly specific to E. Coli but due to evolutionary variation, certain shigella spp. also possess the gene (Molina et al., 2017). In order to conclusively confirm the isolation of E. Coli, the LacY gene was subjected to PCR amplification. This is effective in differentiating E. Coli from Shigella since the latter does not possess certain genes of the lac operon (Ragupathy et al., 2018; Din and Wahid, 2015; Pavlovic et al., 2011; Løbersli et al., 2016; Horakova et al., 2008).

The possibilities of inactive uidA genes, indole negative reactions and 16SrRNA primer-target mismatching due to mutations are also supported by the possibility of DNA damage due to bile salts in the initial chromogenic culture. Bile salts can induce the dinD, micF and osmY genes of E. Coli which are indicators of DNA damage from oxidative stress (Bernstein et al., 1999; Merritt and Donaldson, 2009; Urdaneta and Casadesus, 2017). Additionally, prior to DNA extraction and molecular testing, the samples were stored in 5.00mL of LB broth for 3 months during the quarantine period, therefore, pH variations of the culture may have induced DNA damage or mutations that affected molecular testing. The pH of LB can potentially increase up to 9.0 during prolonged cultures (Sezonov et al., 2007; Mueller, 2007). Further, surface water coliforms are exposed to environmental stresses such as pH and temperature variations, UV radiation exposure, exposure to pesticides and synthetic contaminants, all of which can induce mutagenesis and lead to up/down regulations of several genetic elements (Hughes, 2003; Krishna et al., 2007; Durham et al., 2016; Pullerits et al., 2020).

To test resistance of the selected samples to tetracycline and sulfonamides,

an initial screening by using disc diffusion is an effective technique. The CLSI standard is one of the most commonly used guidelines for ABST using the disc diffusion technique on MHA. The uniform growth of organisms on the agar relies on organisms being in the log phase, therefore overnight cultures ensure that all cells were in the appropriate growth phase (Christenson et al., 2018). Additionally, to ensure uniform concentration and cell abundance, the cultures were diluted and compared with the 0.5McFarland standard. MHA is a non-specific medium, ideal for ABST as it allows uniform diffusion of antibiotics and the simultaneous growth of the organism of interest. The medium does not contain sulfonamide and tetracycline inhibitors such as PABA or thymidine ensuring no interference with results. The non-selectivity of the medium, however, allows the possibility of fungal contaminations and the growth of mixed cultures with both resistant and sensitive strains which can be observed as dispersed colonies growing within the zone of inhibition in S2, S5, S6 (Jorgensen and Hindler, 2006; Hudziki, 2009; Pencheva et al., 2018; Cusack et al., 2019; Mahboob et al., 2019).

E.Coli isolates from the Kelani river have been found to show greater resistance to older antibiotics including tetracycline, however, seasonal variances in the prevalence of genes have been observed. This can account for the absence of sulfonamide resistance and the higher prevalence of tetC genes (S2, S5, S11) in comparison to tetA (S6). Further, the prevalence of sul1 has been found to decrease downstream in the Kelani river (Kumar et al., 2020; Kumar et al., 2020). The tetA is highly prevalent in Sri Lanka and worldwide, however, both tetA and tetC are commonly found in E. Coli (Liyanage and Manage, 2020; Shin et al., 2015). It is possible for organisms to possess multiple resistance genes against the same or varying drugs, therefore, it is possible that the samples also possessed various tetracycline resistance genes (Kumar and Varela, 2012). Further, variations in the zones of inhibition between resistant samples can relate to mutations in tet genes resulting in varying efficacy of the efflux pumps (Linkevicius et al., 2016).

The PCR technique was used several times in the experiment as it is a tool of high specificity and sensitivity, however, if not optimized, anomalies such as smears, primer dimers, faint bands and non-specific amplicons may be present. These can occur due to several parameters, such as high primer or Mg2+ concentrations, impurities in dNTP solutions, non-targeted Taq polymerase activity or degraded DNA due to repetitive thawing. These can be rectified by optimization and troubleshooting techniques such as lowering primer and Mg2+ concentrations, using new dNTP mixtures, using the hot-start PCR technique, keeping DNA samples on ice during PRC mixture preparations (Roux, 2009; Lorenz, 2012; Spibida et al., 2017; Biorad, 2020; Thermofischer Scientific, 2020).

## *CONCLUSION*

It was found that E. Coli isolates obtained from the tube well (S2), and the fishing site (S5 and S11) possessed the tetC resistance gene, whereas the sample obtained from the lake (S6) possessed the tetA resistance gene. This information provides partial evidence of the presence of antibiotic resistant bacteria within the Malabe region, and can be amalgamated with other studies and investigations to assess the prevalence of antibiotic resistance within Sri Lanka, and can be further used provide information to the public regarding the overall quality of water and safety measures to be taken.

## *REFERENCES*


*Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Nisar, M. A., Alvi, R. F., Aslam, M. A., Qamar, M. U., Salamat, M. K. F. and Baloch, Z. (2018) 'Antibiotic resistance: a rundown of a global crisis', Infection and Drug Resistance, 11, pp. 1645–1658, Dovepress [Online]. DOI: 10.2147/IDR.S173867 (Accessed 13 July 2020).*

*Barbas, C.F., Burton, D.R., Scott, J.K. and Silverman, G.J. (2007) 'Quantitation of DNA and RNA', Cold Springs Harbor Protocols, Cold Springs Harbor press [Online]. Available at: http://cshprotocols.cshlp.org/content/2007/11/pdb.ip47.full (Accessed 13 August 2020).*

*Barbau-Piednoir, E., Denayer, S., Botteldoorn, N., Dierick, K., De Keersmaecker, S. C. J. and Roosens, N. H. (2018) 'Detection and discrimination of five E. coli pathotypes using a combinatory SYBR® Green qPCR screening system', Applied Microbiology and Biotechnology, 102(7), pp. 3267–3285, Springer Link [Online]. DOI: 10.1007/s00253-018-8820-0 (Accessed 12 July 2020).*

*Bardal, S.K., Waechter., J.E, and Martin, D.S. (2011), 'Infectious Diseases' in Bardal, S.K., Waechter., J.E, and Martin, D.S. Applied Pharmacology, pp.233-291 [Online] Available at: https://doi.org/10.1016/B978-1-4377-0310-8.00018-X (Accessed 01 April 2020).*

*Berglund, B. (2015) 'Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics', Infection Ecology & Epidemiology, 5(1), pp. 28564, Taylor and Francis [Online]. DOI: 10.3402/iee.v5.28564 (Accessed 09 July 2020).*

*Bernstein, C., Bernstein, H., Payne, C. M., Beard, S. E. and Schneider, J. (1999) 'Bile Salt Activation of Stress Response Promoters in Escherichia coli', Current Microbiology, 39(2), pp. 68–72, Springer Link [Online]. DOI: 10.1007/s002849900420 (Accessed 30 August 2020).*

*Biorad (2020) PCR Troubleshooting. Available at: https://www.bio-rad.com/en-lk/applications-technologies/pcr-troubleshooting?ID=LUSO3HC4S (Accessed 19 August 2020).*

*Capasso, C. and Supuran, C.T. (2019) 'Dihydropteroate Synthase (Sulfonamides) and Dihydrofolate Reductase Inhibitors', in: Bonev, B.B., Brown, N.M. (eds.) Bacterial Resistance to Antibiotics – From Molecules to Man, pp. 163–172 [Online] Available at: https://doi.org/10.1002/9781119593522.ch7 (Accessed 01 April 2020).*

*Chen B, Liang X, Huang X, Zhang T, Li X (2013) 'Differentiating anthropogenic impacts on ARGs in the Pearl River Estuary by using suitable gene indicators' Water Research, 47(8), pp.2811-2820, Science Direct [Online]. DOI:10.1016/j.watres.2013.02.042 (Accessed 19 August 2020).*

*Choshki, A., Sifri, Z., Cennimo, D. and Horng, H. (2019) 'Global contributors to antibiotic resistance', Journal of Global Infectious Diseases, 11(1), pp. 36-42, [Online]. Available at: http://www.jgid.org/article.asp?issn=0974-777X;year=2019;volume=11;issue=1;spage=36;epage=42;aulast=Chokshi (Accessed 15 July 2020).*

*Christenson, J. C., Korgenski, E. K. and Relich, R. F. (2018) 'Laboratory Diagnosis of Infection Due to Bacteria, Fungi, Parasites, and Rickettsiae', in Principles and Practice of Pediatric Infectious Diseases. Elsevier, pp. 1422-1434.e3, Science Direct [Online]. DOI: 10.1016/B978-0-323-40181-4.00286-3 (Accessed 30 August 2020).*

*Collignon, P., Beggs, J.J., Walsh, T.R., Gandra, S. and Laxminarayan, R. (2018) 'Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a*

*univariate and multivariable analysis', The Lancet, 9(2), pp. e398–e405, Planetary Healty [Online]. DOI: https://doi.org/10.1016/S2542-5196(18)30186-4 (Accessed 01 April 2020).*

*Cusack, T. P., Ashley, E. A., Ling, C. L., Roberts, T., Turner, P., Wangrangsimakul, T. and Dance, D. A. B. (2019) 'Time to switch from CLSI to EUCAST? A Southeast Asian perspective', Clinical Microbiology and Infection, 25(7), pp. 782–785 [Online]. DOI: 10.1016/j.cmi.2019.03.016 (Accessed 30 August 2020).*

*Dashti, A. A., Jadaon, M. M., Abdulsamad, A. M. and Dashti, H. M. (2009) 'Heat Treatment of Bacteria: A Simple Method of DNA Extraction for Molecular Techniques', KUWAIT MEDICAL JOURNAL, 41(2), pp. 117-122, Semantics scholar [Online]. Available at: https://www.semanticscholar.org/paper/Heat-Treatment-of-Bacteria%3A-A-Simple-Method-of-DNA-Dashti-Jadaon/8b663a7ad54214f35cd65f624680e465deba191c (Accessed 30 July 2020).*

*Davies, J. and Davies, D. (2010) 'Origins and Evolution of Antibiotic Resistance', Microbiology and Molecular Biology Reviews, 74(3), pp. 417–433, ASM [Online]. DOI: 10.1128/MMBR.00016-10 (Accessed 08 July 2020).*

*Din, A. and Wahid, S. (2014) 'Relationship among Shigella spp. and enteroinvasive Escherichia coli (EIEC) and their differentiation', Brazilian Journal of Microbiology, 45(4), pp. 1131–1138, SciELO [Online]. DOI: 10.1590/S1517-83822014000400002 (Accessed 26 August 2020).*

*Durham, B. W., Porter, L., Webb, A. and Thomas, J. (2016) 'Seasonal influence of environmental variables and artificial aeration on Escherichia coli in small urban lakes', Journal of Water and Health, 14(6), pp. 929–941, IWA Publishing [Online]. DOI: 10.2166/wh.2016.020 (Accessed 30 August 2020).*

*El-Hajj, Z. W. and Newman, E. B. (2015) 'How much territory can a single E. coli cell control?', Microbial Physiology and Metabolism, 6, Frontiers in Microbiology [Online]. DOI: 10.3389/fmicb.2015.00309 (Accessed 15 July 2020).*

*Fatemeh, D., Reza, Z. M., Mohammad, A., Salomeh, K., Reza, A. G., Hossein, S., Maryam, S., Azam, A., Mana, S., Negin, N., Reza, K. A. and Saeed, F. (2014) 'Rapid detection of coliforms in drinking water of Arak city using multiplex PCR method in comparison with the standard method of culture (Most Probably Number)', Asian Pacific Journal of Tropical Biomedicine, 4(5), pp. 404–409, Science Direct [Online]. DOI: 10.12980/APJTB.4.2014C896 (Accessed 19 August 2020).*

*Fernando, A. and Rathnayake, U. (2018) 'Stormwater runoff quality in Malabe, Sri Lanka', Engineering and Applied Science Research, 45 (1), pp.70-73, ThaiJo [Online]. DOI: 10.14456/EASR.2018.9 (Accessed 09 July 2020).*

*Finn, T. J., Shewaramani, S., Leahy, S. C., Janssen, P. H. and Moon, C. D. (2017) 'Dynamics and genetic diversification of Escherichia coli during experimental adaptation to an anaerobic environment', PeerJ Life and Environment, 5, p. e3244 [Online]. DOI: 10.7717/peerj.3244 (Accessed 19 August 2020).*

*Grossman, T.H. (2016) 'Tetracycline Antibiotics and Resistance', Cold Spring Harb Perspect Med, 6 [Online]. Available at: https://doi.org/10.1101/cshperspect.a025387 (Accessed 01 April 2020).*

*Han, T.H., Lee, J.-H., Cho, M.H., Wood, T.K. and Lee, J. (2011) 'Environmental factors affecting indole production in Escherichia coli', Research in Microbiology, 162, pp.108–116, Elsevier [Online]. Available at:*

*https://doi.org/10.1016/j.resmic.2010.11.005 (Accessed 01 April 2020).*

*He, S., Gall, D. L. and McMahon, K. D. (2007) '"Candidatus Accumulibacter" Population Structure in Enhanced Biological Phosphorus Removal Sludges as Revealed by Polyphosphate Kinase Genes', Applied and Environmental Microbiology, 73(18), pp. 5865–5874 American Society for Microbiology [Online]. DOI: 10.1128/AEM.01207-07 (Accessed 19 August 2020).*

*HiMedia (2019) Microbiology. Available at: http://www.himedialabs.com/intl/en/products/100000027/Microbiology (Accessed 15 July 2020).*

*Hofwegen, D.J., Hovde, C.J. and Minnich, S.A. (2016) 'Rapid Evolution of Citrate Utilization by Escherichia coli by Direct Selection Requires citT and dctA', Journal of Bacteriology,198, pp.1022–1034, American Society for Microbiology [Online]. Available at: https://doi.org/10.1128/JB.00831-15 (Accessed 01 April 2020).*

*Horakova, K., Mlejnkova, H. and Mlejnek, P. (2008) 'Specific detection of Escherichia coli isolated from water samples using polymerase chain reaction targeting four genes: cytochrome bd complex, lactose permease, β-d-glucuronidase, and β-d-galactosidase', Journal of Applied Microbiology, 105(4), pp. 970–976, Society for Applied Microbiology, [Online]. DOI: 10.1111/j.1365-2672.2008.03838.x (Accessed 16 August 2020).*

*Hudziki, J., (2009) 'Kirby-Bauer Disk Diffusion Susceptibility Test Protocol', American Society for Microbiology [Online]. Available at: https://asm.org/Protocols/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Pro (Accessed 19 August 2020).*

*Hughes, K. A. (2003) 'Influence of Seasonal Environmental Variables on the Distribution of Presumptive Fecal Coliforms around an Antarctic Research Station', Applied and Environmental Microbiology, 69(8), pp. 4884–4891. American Society for Microbiology [Online]. DOI: 10.1128/AEM.69.8.4884-4891.2003 (Accessed 19 August 2020).*

*Jayaweerasingham, M., Angulmaduwa, S. and Liyanapathirana, V. (2019) 'Knowledge, beliefs and practices on antibiotic use and resistance among a group of trainee nurses in Sri Lanka', BMC Res Notes, 12(601), BMC [Online]. Available at: https://doi.org/10.1186/s13104-019-4640-2 (Accessed 01 April 2020).*

*Jenkins, C., Ling, C. L., Ciesielczuk, H. L., Lockwood, J., Hopkins, S., McHugh, T. D., Gillespie, S. H. and Kibbler, C. C. (2012) 'Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice', Journal of Medical Microbiology, 61(4), pp. 483–488, Microbiology society [Online]. DOI: 10.1099/jmm.0.030387-0 (Accessed 30 August 2020).*

*Jiang, H., Cheng, H., Liang, Y., Yu, S., Yu, T., Fang, J. and Zhu, C. (2019) 'Diverse Mobile Genetic Elements and Conjugal Transferability of Sulfonamide Resistance Genes (sul1, sul2, and sul3) in Escherichia coli Isolates From Penaeus vannamei and Pork From Large Markets in Zhejiang, China', Food Microbiology, 10, Front. Microbiol [Online]. Available at: https://doi.org/10.3389/fmicb.2019.01787 (Accessed 01 April 2020).*

*Jorgensen, J. H., Hindler, J. F., Reller, L. B. and Weinstein, M. P. (2007) 'New Consensus Guidelines from the Clinical and Laboratory Standards Institute for Antimicrobial Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria', Clinical Infectious Diseases, 44(2), pp. 280–286, Oxford Academic [Online]. DOI: 10.1086/510431 (Accessed 30 August 2020).*

*Kim, D.-W., Thawng, C.N., Lee, K., Wellington, E.M.H. and Cha, C.-J. (2019) 'A novel sulfonamide resistance mechanism by two-component flavin-dependent monooxygenase*

*system in sulfonamide-degrading actinobacteria', Environment International, 127, pp. 206–215, Elsevier [Online]. Available at: https://doi.org/10.1016/j.envint.2019.03.046 (Accessed 01 April 2020).*

*Krishna, S., Maslov, S. and Sneppen, K. (2007) 'UV-induced mutagenesis in Escherichia coli SOS response: A quantitative model', PLoS Computational Biology, 3(3), pp.e41, NCBI [Online]. DOI: 10.1371/journal.pcbi.0030041.eor (Accessed 30 August 2020).*

*Kumar, M., Chaminda, G. G. T. and Honda, R. (2020) 'Seasonality impels the antibiotic resistance in Kelani River of the emerging economy of Sri Lanka', npj Clean Water, 3(1), pp.12, Nature [Online]. DOI: 10.1038/s41545-020-0058-6 (Accessed 13 July 2020).*

*Kumar, M., Chaminda, T., Honda, R. and Furumai, H. (2020) 'Vulnerability of urban waters to emerging contaminants in India and Sri Lanka: Resilience framework and strategy', APN Science Bulletin, 9(1), [Online]. DOI: 10.30852/sb.2019.799 (Accessed 13 July 2020).*

*Kumar, M., Ram, B., Sewwandi, H., Sulfikar, Honda, R. and Chaminda, T. (2020) 'Treatment enhances the prevalence of antibiotic-resistant bacteria and antibiotic resistance genes in the wastewater of Sri Lanka, and India', Environmental Research, 183, Elsevier [Online]. Available at: https://doi.org/10.1016/j.envres.2020.109179 (Accessed 01 April 2020).*

*Kumar, M., Sulfikar, Chaminda, T., Patel, A. K., Sewwandi, H., Mazumder, P., Joshi, M. and Honda, R. (2020) 'Prevalence of antibiotic resistance in the tropical rivers of Sri Lanka and India', Environmental Research, 188, pp. 109765, Science Direct [Online]. DOI: 10.1016/j.envres.2020.109765 (Accessed 13 August 2020).*

*Kumar, S. and Varela, M. F. (2012) 'Biochemistry of Bacterial Multidrug Efflux Pumps', International Journal of Molecular Sciences, 13(4), pp. 4484–4495, MDPI [Online]. DOI: 10.3390/ijms13044484 (Accessed 31 August 2020).*

*Larson, A., Hartinger, S. M., Riveros, M., Salmon-Mulanovich, G., Hattendorf, J., Verastegui, H., Huaylinos, M. L. and Mäusezahl, D. (2019) 'Antibiotic-Resistant Escherichia coli in Drinking Water Samples from Rural Andean Households in Cajamarca, Peru', The American Journal of Tropical Medicine and Hygiene, 100(6), pp. 1363–1368, ASTMH [Online]. DOI: 10.4269/ajtmh.18-0776 (Accessed 13 July 2020).*

*Leon, D., D'Alton, S., Quandt, E.M. and Barrick, J.E. (2018) 'Innovation in an E. coli evolution experiment is contingent on maintaining adaptive potential until competition subsides', PLoS Genetics, 14(4) [Online]. Available at: https://doi.org/10.1371/journal.pgen.1007348 (Accessed 01 April 2020).*

*Li, G. and Young, K. D. (2015) 'A new suite of tnaA mutants suggests that Escherichia coli tryptophanase is regulated by intracellular sequestration and by occlusion of its active site', BMC Microbiology, 15(1), pp. 14, BMC [Online]. DOI: 10.1186/s12866-015-0346-3 (Accessed 13 July 2020).*

*Linkevicius, M., Sandegren, L. and Andersson, D. I. (2016) 'Potential of Tetracycline Resistance Proteins To Evolve Tigecycline Resistance', Antimicrobial Agents and Chemotherapy, 60(2), pp. 789–796, American Society for Microbiology [Online]. DOI: 10.1128/AAC.02465-15 (Accessed 30 August 2020).*

*Little, M. S., Pellock, S. J., Walton, W. G., Tripathy, A. and Redinbo, M. R. (2018) 'Structural basis for the regulation of β-glucuronidase expression by human gut Enterobacteriaceae', Proceedings of the National Academy of Sciences, 115(2), pp. E152–E161 [Online]. DOI: 10.1073/pnas.1716241115 (Accessed 15 July 2020).*

*Liyanage, G.Y. and Manage, P.M. (2019) 'Occurrence and distribution of tetracycline resistance determinants and their pollution profile in selected aquaculture environments in Sri Lanka', Journal of the National Science Foundation of Sri Lanka, 47(4), pp. 455 – 465 [Online]. Available at: http://dx.doi.org/10.4038/jnsfsr.v47i4.9301 (Accessed 01 April 2020).*

*Løbersli, I., Wester, A. L., Kristiansen, Å. and Brandal, L. T. (2016) 'Molecular differentiation of Shigella spp. from enteroinvasive E. coli', European Journal of Microbiology and Immunology, 6(3), pp. 197–205, AKJournals [Online]. DOI: 10.1556/1886.2016.00004 (Accessed 26 August 2020).*

*Lorenz, T. C. (2012) 'Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies', Journal of Visualized Experiments, (63), p.3998 [Online]. DOI: 10.3791/3998 (Accessed 26 August 2020).*

*Madsen, H. T. (2014) 'Membrane Filtration in Water Treatment – Removal of Micropollutants', in Søgaard, E.G. Chemistry of Advanced Environmental Purification Processes of Water, pp. 199–248, [Online] DOI: 10.1016/B978-0-444-53178-0.00006-7 (Accessed 15 July 2020).*

*Mahboob, N., Iqbal, H., Ahmed, M., Magnet, M. M. H. and Mamun, K. Z. (2020) 'Disk diffusion Method in Enriched Mueller Hinton agar for determining susceptibility of Candida isolates from various clinical specimens', Journal of Dhaka Medical College, 28(1), pp. 28–33 Bangladesh Journals Online [Online]. DOI: 10.3329/jdmc.v28i1.45753 (Accessed 30 August 2020).*

*Merritt, M. E. and Donaldson, J. R. (2009) 'Effect of bile salts on the DNA and membrane integrity of enteric bacteria', Journal of Medical Microbiology, 58(12), pp. 1533–1541, Microbiology Society [Online]. DOI: 10.1099/jmm.0.014092-0 (Accessed 30 August 2020).*

*Miotto, M., Fonseca, A.A., Barretta, C., da Silva, H.S., Pellizzaro, T., De Dea Lindner, J., Vieira, C.R.W., Parveen, S. and Prudencio, E.S. (2019) 'Development and application of a real-time polymerase chain reaction method for quantification of Escherichia coli in oysters (Crassostrea gigas)', Food Microbiology,77, pp. 85–92 Elsevier [Online]. Available at: https://doi.org/10.1016/j.fm.2018.08.015 (Accessed 01 April 2020).*

*Molina, F., López-Acedo, E., Tabla, R., Roa, I., Gómez, A. and Rebollo, J. E. (2015) 'Improved detection of Escherichia coli and coliform bacteria by multiplex PCR', BMC Biotechnology, 15(1), pp. 48, BMC [Online]. DOI: 10.1186/s12896-015-0168-2 (Accessed 13 July 2020).*

*Monday, S. R., Whittam, T. S. and Feng, P. C. H. (2001) 'Genetic and Evolutionary Analysis of Mutations in the gusA Gene That Cause the Absence of b-Glucuronidase Activity in Escherichia coli O157:H7', The Journal of Infectious Diseases, 184(7), pp.918-921, Oxford Academic [Online]. DOI: https://doi.org/10.1086/323154 (Accessed 15 July 2020).*

*Mueller, E. A., Westfall, C. S. and Levin, P. A. (2019) 'Environmental pH impacts division assembly and cell size in Escherichia coli'. Biorxiv [Preprint]. DOI: 10.1101/747808 (Accessed 30 August 2020).*

*Nguyen, F., Starosta, A.L., Arenz, S., Sohmen, D., Dönhöfer, A. and Wilson, D.N. (2014) 'Tetracycline antibiotics and resistance mechanisms', Biological Chemistry, 395(5), pp.559–575, De Gruyter [Online]. Available at: https://doi.org/10.1515/hsz-2013-0292 (Accessed 01 April 2020).*

*Nicolaou, K.C. and Rigol, S. (2018) 'A brief history of antibiotics and select advances in their synthesis', Journal of Antibiotics, 71, pp. 153–184. Nature [Online]. Available at:*

*https://doi.org/10.1038/ja.2017.62 (Accessed 01 April 2020).*

*Ogura, Y., Seto, K., Morimoto, Y., Nakamura, K., Sato, M. P., Gotoh, Y., Itoh, T., Toyoda, A., Ohnishi, M. and Hayashi, T. (2018) 'Genomic Characterization of β-Glucuronidase–Positive Escherichia coli O157:H7 Producing Stx2a', Emerging Infectious Diseases, 24(12), pp. 2219–2227, CDC [Online]. DOI: 10.3201/eid2412.180404 (Accessed 15 July 2020).*

*O'Neil, M., McPartlin, J., Arthurc, K., Reidel, S. and McMillan, N.D. (2011) 'Comparison of the TLDA with the Nanodrop and the reference Qubit system', Journal of Physics, 307, pp.12-14, IOP Science [Online]. Available at: https://iopscience.iop.org/article/10.1088/1742-6596/307/1/012047/meta (Accessed 13 August 2020).*

*Orozco-Gómez, D.I., Sosa-Hernández, J.E., Gallardo-Navarro, Ó.A., Santana-Solano, J. and Santillán, M. (2019) 'Bistable behaviour and medium-dependent post-translational regulation of the tryptophanase operon regulatory pathway in Echerichia coli', Scientific Reports, 9(5451), Nature [Online]. Available at: https://doi.org/10.1038/s41598-019-41856-0 (Accessed 01 April 2020).*

*Pavlovic, M., Luze, A., Konrad, R., Berger, A., Sing, A., Busch, U. and Huber, I. (2011) 'Development of a duplex real-time PCR for differentiation between E. coli and Shigella spp.: Shigella-E. coli differentiation PCR', Journal of Applied Microbiology, 110(5), pp. 1245–1251, Society for Applied Microbiology [Online]. DOI: 10.1111/j.1365-2672.2011.04973.x (Accessed 13 July 2020).*

*Pencheva, D. V., Ivancheva, K. S., Rumenkina, M. V., Al-Dzhasem, A. M. and Ivanov, I. N. (2018) 'In Search of the Truth about the Quality of Mueller Hinton Agar and Tested Antimicrobial Discs', The Pharmaceutical and Chemical Journal, 5(1), pp.145-152, Research gate [Online]. Available at: https://www.researchgate.net/publication/323692505_In_Search_of_the_Truth_about_the_Quality_of_Mueller_Hinton_Agar_and_Tested_Antimicrobial_Discs (Accessed 19 August 2020).*

*Percival, S. L. and Williams, D. W. (2014) 'Escherichia coli', in Percival, S. L. and Williams, D. W., Gray, N. F., Yates, M. V. and Chalmers, R. M. Microbiology of Waterborne Diseases pp. 89–117 [Online] DOI: 10.1016/B978-0 -12-415846-7.00006-8 (Accessed 19 August 2020).*

*Perry, J. D. and Freydière, A. M. (2007) 'The application of chromogenic media in clinical microbiology: Application of chromogenic media', Journal of Applied Microbiology, 103(6), pp. 2046–2055, SFAM [Online]. DOI: 10.1111/j.1365-2672.2007.03442.x (Accessed 15 July 2020).*

*Perry, J. D. and Freydière, A. M. (2007) 'The application of chromogenic media in clinical microbiology: Application of chromogenic media', Journal of Applied Microbiology, 103(6), pp. 2046–2055, SFAM [Online]. DOI: 10.1111/j.1365-2672.2007.03442.x (Accessed 15 July 2020).*

*Pleguezuelos-Manzano, C., Puschhof, J., Rosendahl Huber, A., van Hoeck, A., Wood, H.M., Nomburg, J., Gurjao, C., Manders, F., Dalmasso, G., Stege, P.B., Paganelli, F.L., Geurts, M.H., Beumer, J., Mizutani, T., Miao, Y., van der Linden, R., van der Elst, S., Garcia, K.C., Top, J., Willems, R.J.L., Giannakis, M., Bonnet, R., Quirke, P., Meyerson, M., Cuppen, E., van Boxtel, R. and Clevers, H. (2020) 'Mutational signature in colorectal cancer caused by genotoxic pks+ E. coli', Nature, 580 [Online]. Available at: https://doi.org/10.1038/s41586-020-2080-8 (Accessed 01 April 2020).*

*Priyadharshana, U., Piyasiri, L.B. and Wijesinghe, C. (2019) 'Prevalence, antibiotic sensitivity pattern and*

*genetic analysis of extended spectrum beta lactamase producing Escherichia coli and Klebsiella spp among patients with community acquired urinary tract infection in Galle district, Sri Lanka', Ceylon Medical Journal, 64(4), pp.140–145. DOI: http://doi.org/10.4038/cmj.v64i4.8990 (Accessed 01 April 2020).*

*Pullerits, K., Ahlinder, J., Holmer, L., Salomonsson, E., Öhrman, C., Jacobsson, K., Dryselius, R., Forsman, M., Paul, C. J. and Rådström, P. (2020) 'Impact of UV irradiation at full scale on bacterial communities in drinking water', npj Clean Water, 3(1), p.11, Nature [Online]. DOI: 10.1038/s41545-020-0057-7.*

*Ragupathi, D. N. K., Sethuvel, M. D. P., Inbanathan, F. Y. and Veeraraghavan, B. (2018) 'Accurate differentiation of Escherichia coli and Shigella serogroups: challenges and strategies', New Microbes and New Infections, 21, pp. 58–62, Science Direct [Online]. DOI: 10.1016/j.nmni.2017.09.003 (Accessed 30 August 2020).*

*Razavi, M., Marathe, N.P., Gillings, M.R., Flach, C.-F., Kristiansson, E. and Joakim Larsson, D.G. (2017) 'Discovery of the fourth mobile sulfonamide resistance gene', Microbiome 5 (160), BMC [Online]. Available at: https://doi.org/10.1186/s40168-017-0379-y (Accessed 01 April 2020).*

*Rezwan, F., Lan, R. and Reeves, P. R. (2004) 'Molecular Basis of the Indole-Negative Reaction in Shigella Strains: Extensive Damages to the tna Operon by Insertion Sequences', Journal of Bacteriology, 186(21), pp. 7460–7465, American Society for microbiology [Online]. DOI: 10.1128/JB.186.21.7460-7465.2004 (Accessed 13 July 2020).*

*Roberts, M.C. (2020) 'Tetracyclines: Mode of Action and their Bacterial Mechanisms of Resistance', in: Bonev, B.B. and Brown, N.M. (eds.) Bacterial Resistance to Antibiotics – From Molecules to Man, pp. 101–124. [Online] Available at: https://doi.org/10.1002/9781119593522.ch5 (Accessed 01 April 2020).*

*Roberts, M.C. and Schwarz, S. (2017) 'Tetracycline and Chloramphenicol Resistance Mechanisms', in Mayers, D.L., Sobel, J.D., Ouellette, M., Kaye, K.S. and Marchaim, D. (Eds.), Antimicrobial Drug Resistance. pp. 231–243. [Online] Available at: https://doi.org/10.1007/978-3-319-46718-4_15 (Accessed 01 April 2020).*

*Roux, K.H. (2009) 'Optimization and Troubleshooting in PCR', Cold Springs Harbor Protocols, 2009(4), Cold Springs Harbor Press [Online]. DOI: 10.1101/pdb.ip66 (Accessed 15 August 2020).*

*Samuel, L. and Plebani, M. (2017) 'Targeting errors in microbiology: the case of the Gram stain', Clinical Chemistry and Laboratory Medicine, 55(3), DeGruyter [Online]. DOI: 10.1515/cclm-2016-0828 (Accessed 15 July 2020).*

*Samuel, L. P., Balada-Llasat, J.-M., Harrington, A. and Cavagnolo, R. (2016) 'Multicenter Assessment of Gram Stain Error Rates', Journal of Clinical Microbiology. Edited by P. Bourbeau, 54(6), pp. 1442–1447, ASM [Online]. DOI: 10.1128/JCM.03066-15 (Accessed 15 July 2020).*

*Sánchez-Clemente, R., Igeño, M. I., Población, A. G., Guijo, M. I., Merchán, F. and Blasco, R. (2018) 'Study of pH Changes in Media during Bacterial Growth of Several Environmental Strains', Proceedings, 2(20), p. 1297, MDPI [Online]. DOI: 10.3390/proceedings2201297 (Accessed 24 July 2020).*

*Sanganyado, E. and Gwenzi, W. (2019) 'Antibiotic resistance in drinking water systems: Occurrence, removal, and human health risks', Science of the Total Environment, 669, pp. 785–797 Elsevier [Online]. Available at: https://doi.org/10.1016/j.scitotenv.2019.03.162 (Accessed 01 April 2020).*

*Sezonov, G., Joseleau-Petit, D. and D'Ari, R. (2007) 'Escherichia coli Physiology in Luria-Bertani Broth', Journal of Bacteriology, 189(23), pp. 8746–8749, American Society for microbiology [Online]. DOI: 10.1128/JB.01368-07 (Accessed 13 July 2020).*

*Shin, S. W., Shin, M. K., Jung, M., Belaynehe, K. M. and Yoo, H. S. (2015) 'Prevalence of Antimicrobial Resistance and Transfer of Tetracycline Resistance Genes in Escherichia coli Isolates from Beef Cattle', Applied and Environmental Microbiology, 81(16), pp. 5560–5566, American Society for Microbiology [Online]. DOI: 10.1128/AEM.01511-15 (Accessed 15 July 2020).*

*Sivri, N., Allen, M.J., Jones, M., Seker, D.Z., Durmus, T., Balci, M. and Balkis, N. (2018) 'Distribution of enteric bacteria by means of GIS and detection of Escherichia coli with uidA gene in Kapıdağ Peninsula of Marmara Sea', Indian Journal of Maritime Science 47(8), ORE [Online]. Available at: https://ore.exeter.ac.uk/repository/handle/10871/34593 (Accessed 01 April 2020).*

*Śpibida, M., Krawczyk, B., Olszewski, M. and Kur, J. (2017) 'Modified DNA polymerases for PCR troubleshooting', Journal of Applied Genetics, 58(1), pp. 133–142, Springer Link [Online]. DOI: 10.1007/s13353-016-0371-4 (Accessed 26 August 2020).*

*Stancik, L. M., Stancik, D. M., Schmidt, B., Barnhart, D. M., Yoncheva, Y. N. and Slonczewski, J. L. (2002) 'pH-Dependent Expression of Periplasmic Proteins and Amino Acid Catabolism in Escherichia coli', Journal of Bacteriology, 184(15), pp. 4246–4258, American Society for microbiology [Online]. DOI: 10.1128/JB.184.15.4246-4258.2002 (Accessed 26 July 2020).*

*Surasinghe, T., Kariyawasam, R., Sudasinghe, H. and Karunarathna, S. (2019) 'Challenges in Biodiversity Conservation in a Highly Modified Tropical River Basin in Sri Lanka', Water, 12(1), pp. 26 MDPI [Online]. DOI: 10.3390/w12010026 (Accessed 15 July 2020).*

*Thermofischer Scientific (2020) PCR Troubleshooting Guide. Available at: https://www.thermofisher.com/lk/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-troubleshooting.html (Accessed 19 August 2020).*

*Toner, E., Adalja, A., Gronvall, G. K., Cicero, A. and Inglesby, T. V. (2015) 'Antimicrobial Resistance Is a Global Health Emergency', Health Security, 13(3), pp. 153–155, Mary Anne Leibert Inc. [Online]. DOI: 10.1089/hs.2014.0088 (Accessed 15 July 2020).*

*Urdaneta, V. and Casadesús, J. (2017) 'Interactions between Bacteria and Bile Salts in the Gastrointestinal and Hepatobiliary Tracts', Gastroenterology, 4, p.163, Frontiers in Medicine [Online]. DOI: 10.3389/fmed.2017.00163 (Accessed 26 July 2020).*

*Usman, H.M., Abdulkadir, N., Gani, M. and Maiturare, M. (2017) 'Bacterial Pigments and its Significance', MOJ Bioequivalence & Bioavailability, 4(3), MedCrave [Online]. DOI: 10.15406/mojbb.2017.04.00073 (Accessed 13 July 2020).*

*Wang, X., Jordan, I. K. and Mayer, L. W. (2015) 'A Phylogenetic Perspective on Molecular Epidemiology', in Tang, Y., Sussman, M., Liu, D., Poxton, I. and Schwartzman, J. Molecular Medical Microbiology, pp. 517–536 [Online] DOI: 10.1016/B978-0-12-397169-2.00029-9 (Accessed 08 August 2020).*

*Watts, J., Schreier, H., Lanska, L. and Hale, M. (2017) 'The Rising Tide of Antimicrobial Resistance in Aquaculture: Sources, Sinks and Solutions', Marine Drugs, 15(6), pp.158, MDPI [Online]. Available*

*at: https://doi.org/10.3390/md15060158 (Accessed 01 April 2020).*

*Winand, R., Bogaerts, B., Hoffman, S., Lefevre, L., Delvoye, M., Van Braekel, J., Fu, Q., Roosens, N. H., De Keersmaecker, S. C. and Vanneste, K. (2019) 'Targeting the 16S rRNA Gene for Bacterial Identification in Complex Mixed Samples: Comparative Evaluation of Second (Illumina) and Third (Oxford Nanopore Technologies) Generation Sequencing Technologies', International Journal of Molecular Sciences, 21(1), pp.298, MDPI [Online]. DOI: 10.3390/ijms21010298 (Accessed 31 August 2020).*

*Witzke, M. C., Gullic, A., Yang, P., Bivens, N. J., Adkins, P. R. F. and Ericsson, A. C. (2020) 'Influence of PCR cycle number on 16S rRNA gene amplicon sequencing of low biomass samples', Journal of Microbiological Methods, 176, p.106033, Science Direct [Online]. DOI: 10.1016/j.mimet.2020.106033 (Accessed 31 August 2020).*

*World Health Organization (2020) Antibiotic Resistance. Available at: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance (Accessed 15 July 2020).*

*Yousef, F., Mansour, O. and Herbali, J. (2018) 'Sulfonamides: Historical Discovery Development', In-vitro In-vivo In-silico Journal, 1(1), Open Access Pub [Online]. Available at: https://openaccesspub.org/iiij/article/749 (Accessed 01 April 2020).*