APPENDIX I

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

NHRC APPROVAL

Ref. No.: 1015

29 November 2018

Ms. Manita Tumbahamgphe
Principal Investigator
Central Campus of Technology

Ref: **Approval of thesis proposal** entitled **Comparative study of biofilm producing and non-producing Escherichia coli isolated from urine samples of patients visiting a tertiary care hospital of Morang, Nepal**

Dear Ms. Tumbahamgphe,

It is my pleasure to inform you that the above-mentioned proposal submitted on **20 September 2018** (**Reg. no. 622/2018**) has been approved by Nepal Health Research Council (NHRC) National Ethical Guidelines for Health Research in Nepal, Standard Operating Procedures Section 'C' point no. 6.3 through Expedited Review Procedures.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol. Expiration date of this proposal is **December 2018.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and **submit progress report in between and full or summary report upon completion.**

As per your thesis proposal, the total research budget is **NRs 18,000** and accordingly the processing fee amounts to **NRs 1,000.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

Thanking you,

Nirbhay Kumar Sharma
Deputy Chief Admin Officer

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APPENDIX II

Materials and Equipments

A. List of Materials

Glass Wares

Beakers
Slides
Micropipette
Glass rod
Measuring cylinder
Durham's tubes
Culture tubes
Test tubes
Conical flask
Pipettes
Micropipette tips

Miscellaneous

Bacteriological loop
Sterile cotton swabs
Marker
Tube holder
Staining rack
Gloves
Labeling stickers
Bunsen burner
Forceps
Tissue paper
96-well ELISA plates
Spirit lamp
Soaps
Micro-centrifuge tubes

B. Equipments

Auto clave
Microscope
Incubator
Colony counter
Hot air oven
Water distillation plant
Refrigerator
ELISA reader

Chemicals and Reagents

Crystal violet
Alcohol
Crystal violet solution 0.1%
Hydrochloric acid
Microscope oil
Kovac's reagent
40% KOH
3% H_2O_2
Gram's iodine
Safranin
PBS
Sodium hydroxide
Lysol
Methyl red
α-napthol

Media (HiMedia Company)

MacConkey Agar
Muller Hinton Agar
Eosine Methylene Blue Agar
Starch Agar

Antibiotics (HiMedia Company)

Amoxicillin	Ampicillin	Cefotaxime
Ceftriaxone	Cephoxitin	Chloramphenicol
Ciprofloxacin	Cotrimoxazole	Gentamycin
Nalidixic acid	Norfloxacin	Ofloxacin
Tetracycline	Trimethoprim	

APPENDIX III

Composition of different types of media

1. CLED Agar

Ingredients	Amount
Lactose	10 gm
Pancreatic digest of gelatin	4 gm
Pancreatic digest of casein	4 gm
Beef extract	3 gm
L-cystine	0.128 g
Bromothymol blue	0.02 gm
Agar	15 gm

Final pH 7.2 +/- 0.2 at 25°C.

2. Lysogeny broth

Ingredients	Gm/L
Tryptone	10.00
Yeast extract	5.00
NaCl	10
pH	7.5 to 8

3. Muller Hinton Agar

Ingredients	Gm/L
Beef extract	300.0
Casein acid hydrosylate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25°C)	7.4±0.2

38 grams of the medium was suspended in 1000ml and boiled to dissolve completely. The medium was then autoclaved at 15 lbs at 121°C for 15 minutes.

4. PBS

For one litre of 1X PBS: Add 8gm of NaCl. 0.2gm of KCl
1.44gm of $Na2HPO_4$.0.2gm of KH_2PO_4. Adjust the pH to 7.4 with HCL. Add distilled water to total volume of 1 litre and sterilize. Store at room temperature.

6.Tryptic Soya Broth (TSB)

Ingredients	Gm/L
Pancreatic digest of casein	17.0 gm
Sodium chloride	5.0 gm
Papaic digest of soyabean meal	3.0 gm
Dextrose	2.5 gm
Dipotassium phosphate	2.5 gm
Final pH	7.3±0.2 at 25°C

APPENDEX IV

Biochemical tests procedure

1. Grams staining procedure

1. Applying a primary **stain** (crystal violet) to a heat-fixed smear of a bacterial culture.
2. The addition of iodide, which binds to crystal violet and traps it in the cell.
3. Rapid decolorization with ethanol or acetone.
4. Counterstaining with safranin.

2. Catalase test procedure

Place a microscope slide inside a petri dish. Keep the petri dish cover available. The use of a petri dish is optional as the slide catalase can be properly performed without it. Using a sterile inoculating loop or wooden applicator stick, collect a small amount of organism from a well-isolated 18- to 24-hour colony and place it onto the microscope slide. Be careful not to pick up any agar. Using a dropper or Pasteur pipette, place 1 drop of 3% H_2O_2 onto the organism on the microscope slide. Do not mix. Immediately cover the petri dish with a lid to limit aerosols and observe for immediate bubble formation (O2 + water = bubbles). Observing for the formation of bubbles against a dark background enhances readability. Positive reactions are evident by immediate effervescence (bubble formation) (Fig. 1). Place microscope slide over a dark background and use a magnifying glass or microscope to observe weak positive reactions. If using a microscope, place a cover slip over the slide and view under 40x magnification. No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction.

3. Indole Test

1. Obtain a culture of Escherichia coli.
2. Obtain a sterile tube of tryptone broth.
3. Using a wax pencil label the tryptone broth tube with the initials of the bacteria and the initials of someone in the group.
4. Using an inoculating loop and aseptic technique, transfer a loopful of bacteria into the tryptone broth.
5. Be sure to shake the loop in the broth and touch it to the side of the tube toremove excess broth.
6. Place both tubes in a test tube rack and place them in the incubator.
7. Obtain the inoculated tryptone broth tubes from the incubator
8. Add 10 drops of Kovac's reagent to the tube inoculated with *Escherichia coli*.
9. Allow the tube to stand for 5 minutes.
10. Repeat the procedure with the tube inoculated with *Enterobacter aerogenes*.
11. A red ring will form at the top of the broth if it positive for indole production.
13. A yellow or green ring will form at the top of the broth if it is negative for indole production.

4. Methyl Red Test

1. Obtain a culture of *Escherichia coli*.
2. Obtain a sterile tube of dextrose broth.
3. Using a wax pencil label the dextrose broth with the initials of the bacteria and the initials of someone in the group.
4. Using an inoculating loop and aseptic technique, transfer a loopful of bacteria into the dextrose broth.
5. Be sure to shake the loop in the broth and touch the loop to the side of the tube to remove excess broth.
6. Place both tubes in a test tube rack and place them in the incubator.
7. Obtain the inoculated dextrose broth tubes from the incubator.
8. Add 5 drops of Methyl red to the tube inoculated with *Escherichia coli*.
9. Roll the tube between your hands to mix.
10. Repeat the procedure with the tube inoculated with *Enterobacter aerogenes*.
11. A red color indicates a positive test for acid production.
12. A yellow color indicates a negative test for acid production.
13. An orange color indicates an inconclusive or negative test for acid production.

5. Citrate Utilization

1. Obtain a culture of *Escherichia coli*.
2. Obtain a sterile citrate slant.
3. Using a wax pencil label the citrate slant with the initials of the bacteria and the initials of someone in the group.
4. Using an inoculating needle and aseptic technique, obtain a sample of the bacteria.
5. Insert the needle into the butt of the citrate and as the needle is pulled out of the butt, streak the slant in a zigzag pattern.
6. Place both tubes in a test tube rack and place them in the incubator.
7. Obtain the inoculated Simmon's citrate slants from the incubator.
8. Observe the slant inoculated with *Escherichia coli.*
9. Observe the slant inoculated with *Enterobacter aerogenes.*
10. A blue color of the agar indicates a positive test for citrate utilization.
11. A green color of the agar indicates a negative test for citrate utilization.

6. Voges-Proskauer

1. Prior to inoculation, allow medium to equilibrate to room temperature.
2. Using organisms taken from an 18-24 hour pure culture, lightly inoculate the medium.
3. Incubate aerobically at 37 degrees C. for 24 hours.
4. Following 24 hours of incubation, aliquot 2 ml of the broth to a clean test tube.
5. Re-incubate the remaining broth for an additional 24 hours.
6. Add 6 drops of 5% alpha-naphthol, and mix well to aerate.
7. Add 2 drops of 40% potassium hydroxide, and mix well to aerate.
8. Observe for a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.

7. Carbohydrate fermentation test

Materials Required:

1. Phenol Red Carbohydrate Fermentation Broth.
2. Bacterial culture.
3. Inoculation loop.
4. Incubator (37^0 C).

Procedure:

I. Preparation of Carbohydrate Fermentation Broth

1. Weigh and dissolve trypticase, Sodium chloride, and Phenol red in 100 ml distilled water and transfer into conical flasks.
2. Add 0.5% to 1% of desired carbohydrate into all flasks.
3. Insert inverted Durham tubes into all tubes, the Durham tubes should be fully filled with broth.
4. Sterilize at 115^0 C for 15 minutes.
5. **Important**: Do not overheat the Phenol red Carbohydrate fermentation broth. The overheating will result in breaking down of the molecules and form compounds with a characteristic color and flavour. The process is known as caramelization of sugar (the browning of sugar).
6. Transfer the sugar into screw capped tubes or fermentation tubes and label properly.

Ingredients of the Fermentation Broth:

1. Trypticase: 1g
2. Carbohydrate: 0.5g
3. Sodium Chloride: 0.5g
4. Phenol red : 0.0189mg

*Autoclave at 115^o C for 15 minutes

II. Inoculation of Bacterial Culture into the Phenol Red Carbohydrate Broth

1. Aseptically inoculate each labeled carbohydrate broth with bacterial culture. (Keep uninoculated tubes as control tubes).
2. Incubate the tubes at 18-24 hours at 37^oC.
3. Observe the reaction.

Precautions:

- After inoculation into a particular sugar, sterilize the loop in order to avoid cross contamination of the tube with other sugars.
- Keep uninoculated sugar tubes as control tubes.
- Do not use the tubes with Durham tubes that are partially filled or with bubbles.
- Over incubation will help the bacteria to degrade proteins and will result give false positive results.

8. Starch hydrolysis test

1. Using a sterile technique, make a single streak inoculation of organism to be tested into the centre of labelled plate.
2. Incubate the bacterial inoculated plates for 48 hours at 37°C.
3. Following incubation, flood the surface of the plates with iodine solution with a dropper for 30 seconds.
4. Pour off the excess iodine.

5. Examine for the clear zone around the line of bacterial growth.

9. Triple Sugar Iron Agar (TSI) Test

1. With a sterilized straight inoculation needle touch the top of a well-isolated colony.
2. Inoculate TSI Agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant.
3. Leave the cap on loosely and incubate the tube at 35°C in ambient air for 18 to 24 hours.

Interpretation of Triple Sugar Iron Agar Test

1. If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in butt and in the slant. Some organisms generate gases, which produces bubbles/cracks on the medium.
2. If lactose is not fermented but the small amount of glucose is, the oxygen-deficient butt will be yellow (remember that butt comparatively have more glucose compared to slant i.e. more media more glucose), but on the slant the acid (less acid as media in slant is very less) will be oxidized to carbon dioxide and water by the organism and the slant will be red(alkaline or neutral pH).
3. If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of production of ammonia from the oxidative deamination of amino acids (remember peoptone is a major constituent of TSI Agar).
4. If H2S is produced, the black color of ferrous sulfide is seen.

APPENDIX V

Statistical Analysis

Biofilm * MDR

Crosstab

Count

		MDR		Total
		No	Yes	
Biofilm	Medium	6	20	26
	Strong	3	16	19
	Weak	9	6	15
Total		18	42	60

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.849[a]	2	.012
Likelihood Ratio	8.449	2	.015
N of Valid Cases	60		

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 4.50.

Result: P <0.05 Test is statistically significant.

Cefotaxime

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	24.307[a]	1	.000		
Continuity Correction[b]	22.540	1	.000		
Likelihood Ratio	25.203	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	120				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 29.50.

b. Computed only for a 2x2 table

Nalidixic acid

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	12.037[a]	1	.001		
Continuity Correction[b]	10.803	1	.001		
Likelihood Ratio	12.247	1	.000		
Fisher's Exact Test				.001	.000
N of Valid Cases	120				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 29.50.
b. Computed only for a 2x2 table

Norfloxacin

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.300[a]	1	.584		
Continuity Correction[b]	.133	1	.715		
Likelihood Ratio	.300	1	.584		
Fisher's Exact Test				.715	.358
N of Valid Cases	120				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 29.50.
b. Computed only for a 2x2 table

Microtitre ,TM and CRA

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	40.845[a]	1	.000		
Continuity Correction[b]	38.544	1	.000		
Likelihood Ratio	43.555	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	120				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 29.50.
b. Computed only for a 2x2 table

Co –trimaxole

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.635[a]	1	.018		
Continuity Correction[b]	4.801	1	.028		
Likelihood Ratio	5.680	1	.017		
Fisher's Exact Test				.028	.014
N of Valid Cases	120				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 29.50.

b. Computed only for a 2x2 table

Biofilm and non-Biofilm producing Resistant

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	28.041[a]	1	.000		
Continuity Correction[b]	26.141	1	.000		
Likelihood Ratio	29.252	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	120				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 29.50.

b. Computed only for a 2x2 table

APPENDIX VI

Formulas

The sensitivity, specificity, PPV, NPV values were calculated by using the following formula:

Sensitivity = (TP/TP+FN) × 100%

Specificity = (TN/TN+FP) × 100%

PPV = (TP/TP+FP) × 100%

NPV = (TN/TN+FN) × 100%

PPV= Positive predictive value

NPV= Negative predictive value

TP= True positive

TN= True negative

FP= False positive

FN= False negative