APPENDIX: I

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 3127

20 June 2018

Mr. Jenish Shakya
Principal Investigator
Central Campus of Technology

Ref: **Approval of thesis proposal** entitled **Antibiogram of biofilm producing and non-producing CA-MRSA isolated from potential risk population of Dharan, Nepal**

Dear Mr. Shakya,

It is my pleasure to inform you that the above-mentioned proposal submitted on **28 May 2018** (**Reg. no. 297/2018**) 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 **September 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 17,000** 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,

Prof. Dr. Anjani Kumar Jha
Executive Chairperson

APPENDIX: II

Materials and Equipments

A. List of Materials

1. Glass wares

Beaker
Conical flask
Petri plates
Measuring cylinder
Test tubes
Glass rod
Micropipette
Micropipette tips
Glass slides
Dolly rods

2. Miscellaneous

Bunsen burner
Gloves
Hi-media sterile cotton swabs
Bacteriological loop
Forceps
Permanent marker
Soaps
Labeling tags
96-well ELISA plates
Ice box

3. Equipments

Autoclave
Hot air oven
Incubator
Refrigerator
Compound Microscope
ELISA Reader
Weight Balance

4. Chemical and Reagents

Crystal Violet (CV) solution 0.1%
30% acetic acid
Methylene blue
PBS
Ethanol 1N
Lysol
Plasma
Microscope oil
Sodium Chloride
3% H_2O_2

5. Microbiological Media (HiMedia)

Mannitol Salt Agar (MSA)
Muller Hinton Agar (MHA)
Trypticase Soya Broth (TSB)
Nutrient Broth (NB)
Nutrient Agar (NA)
Agar

6. Antibiotics Discs

HiMedia

Ampicillin (AMP, 10mcg)
Amoxicillin (AMX, 10mcg)
Cefoxitin (CX, 30mcg)
Cefotaxime (CTX, 30mcg)
Chloramphenicol (C, 30mcg)
Ciprofloxacin (CIP, 5mcg)
Co-Trimoxazole (COT, 25mcg)
Erythromycin (E, 15mcg)
Gentamicin (GEN, 10mcg)
Norfloxacin (NX, 10mcg)
Ofloxacin (OF, 5mcg)
Teicoplanin (TEI, 30mcg)
Tetracycline (TE, 30mcg)
Trimethoprim (TR, 5mcg)
Vancomycin powder (HiMedia, India)

APPENDIX: III

Bacteriological media

Composition and preparation of different types of media

1. Mannitol Salt Agar (MSA)

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	5.000
Pancreatic digest of casein	5.000
Beef extract	1.000
Sodium chloride	75.000
D-Mannitol	10.000
Phenol red	0.025
Agar	15.000
pH after sterilization (at 25°C)	7.4±0.2

2. Nutrient Broth (NB)

Peptone	5.0g
Sodium chloride	5.0g
Beef Extract	1.5g
Yeast Extract	1.5g
Final p^H	7.4±0.2

1.3 gm of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121°C for 15 minutes.

3. Muller Hinton Agar (MHA)

Beef infusion Broth	300.0g
Casein Acid Hydrolysate	17.0g
Starch	1.0g
Agar	17.0g
Final pH	7.0±0.2

3.8 gm of media was suspended in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121°C for 15 minutes. It was then poured while at 45-48°C into sterile petriplates in 25 ml quantity.

4. Phosphate Buffer Solution (PBS)

For 1 liter of 1X PBS: Add 8gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na2HPO4, 0.2 gm of KH2PO4, Adjust the pH to 7.4 with HCl. Add distill water to total volume of 1Liter and sterilize. Store at room temperature.

5. Trypticase Soya Broth (TSB)

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 ºC

6. Congo Red Agar Media

Brain heart infusion broth (HiMedia, Mumbai, India) - 37 g/L
Sucrose - 50 g/L
Agar No. 1 (HiMedia, Mumbai, India) - 10 g/L
Congo Red indicator (HiMedia, Mumbai, India) - 8 g/L

APPENDIX: IV

Composition and preparation of different reagents

1. Gram staining reagents

i. Crystal violet Gram stain

Crystal violet	20g
Ammonium oxalate	9g
Ethanol or methanol, absolute	95ml
Distilled water	1 litre

Preparation:

Crystal violet is weighed and transferred to a clean bottle and absolute ethanol is added and mixed until dye is completely dissolved.

Ammonium oxalate is weighed and dissolved in about 200 ml of distilled water. Then it was added to the stain and total volume is made 1 litre by adding distilled water and mixed well.

ii. Iodine Solution

Potassium iodide	1.5g
Iodine	1.0g
Distilled water	150ml

Preparation:

Potassium iodide is weighed and transferred to a clean bottle 30-40 ml of distilled water is added to Potassium iodide and mixed until it is fully dissolved.

Iodine is weighed and added to potassium iodide solution and mixed well.

Final volume is made 150ml by adding distilled water and mixed well.

iii. Acetone-alcohol decolorizer

Acetone	500ml
Ethanol (absolute)	475ml
Distilled water	25ml

To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then 500 ml acetone was added and mixed well.

iv. Counterstain solution

Safranine	10gm
Distilled water	1 lit

In a piece of clean paper, 10 gm of safranine was weighed and transferred to a clean bottle. Then after, 1 liter distilled water was added to the bottle and mixed well until safranine dissolves completely.

v. Catalase reagent (To make 100 ml)

Hydrogen peroxide solution	3ml
Distilled water	97ml

Preparation:

To 97 ml distilled water, 3 ml of hydrogen peroxide solution was added and mixed well.

APPENDIX: V

Procedure of different biochemical tests

1. Gram's strain: (Mackie and McCartney Vol.2., 14th edition).

Isolated colony selected for staining:

1. Smear was made from pure culture by emulsifying a colony in normal saline and heat fixed.
2. Smear flooded with crystal violet for 1 mint.
3. Wash with water
4. Add Gram's Iodine for 1minute.
5. Wash with water.
6. Decolorize with absolute alcohol for 10-15secs.
7. Wash with water
8. Flood with saffranin for 1minute.
9. Wash with water, blot dry and examine under oil immersion objective of the microscope.

2. Catalase test

1. A small amount of isolated colony from pure culture was transferred to the surface of clean dry glass slide.
2. A drop of 3% H_2O_2 was placed onto the inoculum.
3. The evolution of oxygen bubbles was recorded immediately.
4. The slide was then discarded into a disinfectant.

3. Coagulase test

I. Slide test (to detect bound coagulase)

1. A drop of physiological saline was placed on end of a slide and colony of test organism was emulsified in each of the drops to make two thick suspensions.
2. A drop of plasma was added to one of the suspensions and mixed gently. It was looked for clumping of the organism within 10 seconds. But no plasma was added to second suspension. This is used to differentiate any granular appearance of the organism from the coagulase clumping.

II. Tube test (to detect free coagulase)

1. The plasma was diluted 1 in 10 physiological saline (mixing 0.2 ml of plasma with 1.8 ml of saline)
2. 3 tubes were taken and labeled as:

T = test organism (18-24 hour broth culture),
P = Positive control (*S. aureus* broth culture),
N = Negative control (sterile broth).

3. 0.5 ml of diluted plasma was pipetted into each tube.
4. About 5-5 drops each of test organism, *S. aureus* culture, and sterile broth was added to the tubes labeled 'T', 'P' and 'N' respectively.
5. After mixing gently, 3 tubes were incubated at 37^oC. It was examined for clotting after 1 hour. If no clotting occurs tubes were examined at 30 minutes intervals for up to 6 hours.

APPENDIX: VI

Antibiotic Susceptibility Test (Kirby-Bauer's Disc Diffusion Method)

In vitro susceptibility of the pure bacterial species to fifteen different antibiotics was determined using Kirby- Bauer disk diffusion technique using Muller-Hinton agar and antibiotic discs as described by the National Committee for Clinical Laboratory Standards (CLSI, 2006). One ml of each bacterial isolates prepared directly from an overnight agar plates adjusted to 0.5 McFarland Standard was inoculated using sterile swab into each of the Petri-dishes containing Mueller-Hinton agar and were allowed to stand for 30 minutes for pre-diffusion of the inoculated organisms.

Antibiotic discs were seeded into the petri dishes containing Mueller-Hinton agar (MHA) for each bacterial isolates. The AST of the isolates towards various antimicrobial discs was done by modified Kirby-Bauer M2-A9 disc diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI) using MHA as follows:

1. MHA was prepared and sterilized as instructed by the manufacturer.
2. The pH of the medium was adjusted to 7.2-7.4 and the depth of the medium at 4mm (about 25 ml per plate) was maintained in petri dish.
3. Using a sterile wire loop, a single isolated colony whose susceptibility pattern is to be determined was touched and inoculated into MHB tube and was incubated at 37oC for 2-4 hrs.
4. After incubation, the turbidity of the suspension was matched with the McFarland standard tube number 0.5 (which is equivalent to 10 to power 4 organisms).
5. Using a sterile swab, an MHA plate was inoculated with the matched suspension using a carpet culture technique.
6. The plate was then allowed to stand for 20-30 minutes for the pre-diffusion of the inoculated organisms.
7. Using clean and sterile forceps, the above mentioned antibiotic discs (6 mm) were placed on the MHA. The discs were placed at the considerable distance

apart from each other on a 90 mm Petri-dish. Then the plate was incubated at 37 ºC for 24 hrs.

8. After incubation, the plates were observed for zone of inhibition and the diameters of inhibition zones were measured in millimeters (mm). The measurement was interpreted as sensitive and resistant according to the manufacture's standard zone size interpretative manual of CLSI (2006).

The percentage resistance was calculated using the formula *PR=a/b×100, where 'PR'* was percentage resistance, '*a'* was the number of resistant isolates and '*b'* was the number of isolates tested with the antibiotic. The percentage sensitivity was calculated using the formula *PS=c/d×100, where 'PS'* was percentage sensitivity, '*c'* was the number of sensitive isolates and '*d'* was the number of isolates tested with the antibiotic.

B. Antibiotic disc used and procedure of susceptibility test

1. Antibiotic disc used

Antibiotics used	Symbol	Disc Content (mcg)	Diameter of Zone of inhibition (mm)		
			Resistant	Intermediate	Sensitive
Ampicillin	AMP	10	28	-	29
Amoxicillin	AMX	10	19	-	20
Cefoxitin	CX	30	21	-	22
Cefotaxime	CTX	30	14	15-22	23
Chloramphenicol	C	30	12	13-17	18
Ciprofloxacin	CIP	5	15	16-20	21
Cotrimoxazole	COT	25	10	11-15	16
Erythromycin	E	15	13	14-22	23
Gentamicin	GEN	10	12	13-14	15
Norfloxacin	NX	10	12	13-16	17
Ofloxacin	OF	5	12	13-15	16
Teicoplanin	TEI	30	10	11-13	14
Tetracycline	TE	30	14	15-18	19
Trimethoprim	TR	5	14	14-16	17

Minimum Inhibitory Concentration Interpretative Criteria

The interpretive criteria for vancomycin and *S. aureus* were lowered to ≤ 2 µg/mL for susceptible, 4 to 8 µg/mL for intermediate, and ≥ 16 µg/mL for resistant. (M07-A9-2012)

1) Susceptible – The isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

2) Intermediate – a category that includes isolates with antimicrobial agent minimal inhibitory concentrations that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates.

NOTE: The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g., quinolones and β-lactams in urine) or when a higher than normal dosage of a drug can be used (e.g., β-lactams). This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

3) Resistant – The isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate minimal inhibitory concentrations that fall in the range in which specific microbial resistance mechanisms (e.g., βlactamases) are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

(Source: CLSI M07-A9, 2012).

APPENDIX: VII

1. Biofilm formation and MDR pattern of MRSA isolates

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	409.839[a]	4	.000
Likelihood Ratio	497.853	4	.000
N of Valid Cases	400		

a. 3 cells (33.3%) have expected count less than 5. The minimum expected count is .46.

$H_{0:}$ Biofilm formation has no significant effect on Multi Drug Resistance of MRSA.
H_1: Biofilm formation has significant effect on Multi Drug Resistance of MRSA.
Since , $p< 0.01$ we fail to accept Null Hypothesis
Conclusion: Test is statistically significant. Biofilm formation has significant effect on Multi Drug Resistance of MRSA.

2. Age and Prevalence of MRSA

ANOVA(ONE WAY)

Age_yrs

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.612	1	5.612	.055	.816
Within Groups	40773.162	396	102.963		
Total	40778.774	397			

Test is not statistically significant.

3. Gender vs MRSA Prevalence

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.277[a]	1	.599		
Continuity Correction[b]	.174	1	.676		
Likelihood Ratio	.277	1	.599		
Fisher's Exact Test				.664	.338
N of Valid Cases	400				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 58.43.
b. Computed only for a 2x2 table

Test is not statistically significant.

4. Work duration vs prevalence of MRSA

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	34.336[a]	32	.356
Likelihood Ratio	40.193	32	.152
N of Valid Cases	400		

a. 41 cells (62.1%) have expected count less than 5. The minimum expected count is .62.

Test is not statistically significant

5. Biofilm and Ampicillin Drug Resistance

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	400.000[a]	3	.000
Likelihood Ratio	493.669	3	.000
N of Valid Cases	400		

a. 0 cells (0.0%) have expected count less than 5.

Test is statistically significant

6. Skin infection history vs MRSA prevalence

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	11.849[a]	1	.001		
Continuity Correction[b]	10.611	1	.001		
Likelihood Ratio	10.910	1	.001		
Fisher's Exact Test				.001	.001
N of Valid Cases	400				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 11.69.

b. Computed only for a 2x2 table

Test is statistically significant

7. Nasal and Hand Source and MRSA Prevalence

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.211[a]	1	.013		
Continuity Correction[b]	5.682	1	.017		
Likelihood Ratio	6.238	1	.013		
Fisher's Exact Test				.017	.008
N of Valid Cases	400				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 61.50.

b. Computed only for a 2x2 table

Test is statistically significant

8. Biofilm formation with Ciprofloxacin Drug

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.052[a]	1	.044		
Continuity Correction[b]	2.637	1	.104		
Likelihood Ratio	4.441	1	.035		
Fisher's Exact Test				.058	.050
N of Valid Cases	123				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.41.

b. Computed only for a 2x2 table

9. Prevalence of MRSA and regular exposure to waste

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7.349[a]	1	.007		
Continuity Correction[b]	6.743	1	.009		
Likelihood Ratio	7.214	1	.007		
Fisher's Exact Test				.009	.005
N of Valid Cases	400				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 42.13.

Test is statistically significant

10. Prevalence of MRSA with Marital Status

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.070[a]	1	.791		
Continuity Correction[b]	.022	1	.883		
Likelihood Ratio	.070	1	.791		
Fisher's Exact Test				.816	.444
N of Valid Cases	400				

Test is not statistically significant

11. MIC of vancomycin to MRSA isolates from hand and nasal sample

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.225[a]	1	.635		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.228	1	.633		
Fisher's Exact Test				1.000	.595
N of Valid Cases	9				

a. 4 cells (100.0%) have expected count less than 5. The minimum expected count is 1.33.

Test is not statistically significant

12. MIC of Vancomycin to total MRSA isolates

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	101.000[a]	4	.000
Likelihood Ratio	11.220	4	.024
N of Valid Cases	101		

a. 5 cells (55.6%) have expected count less than 5.

13. ANOVA (Two Way)

Tests of Between-Subjects Effects

Dependent Variable: Sensitivity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26427.942[a]	13	2032.919	35.577	.000
Intercept	59921.503	1	59921.503	1048.646	.000
Antibiotics	26427.942	13	2032.919	35.577	.000
Error	799.985	14	57.142		
Total	87149.430	28			
Corrected Total	27227.927	27			

a. R Squared = .971 (Adjusted R Squared = .943)

Result: Test is statistically significant

APPENDIX: VIII

Formulas:

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

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100\%$$

$$\text{Specificity} = \frac{TN}{TN+FP} \times 100\%$$

$$\text{PPV} = \frac{TP}{TP+FP} \times 100\%$$

$$\text{NPV} = \frac{TP}{TP+FP} \times 100\%$$

PPV = positive predictive value

NPV = negative predictive value

TP = true positive

TN = true negative

FP = false positive

FN = false negative