

APPENDIX-I

QUESTIONNAIRE OF PREVALENCE OF URINARY TRACT INFECTION IN DIABETIC PATIENT AND ASSESSMENT OF RISK FACTORS OF URINARY TRACT INFECTION”

DATE-.....

SAMPLE NO-.....

NAME-AGE-WEIGHT-.....

ADDRESS.....SEX.....

EDUCATION-.....BLOOD PRESSURE-.....

OCCUPATION-.....BLOOD GROUP-.....

MARITAL STATUS-.....

NUMBER OF PREGNANCY-

BLOOD SUGAR LEVEL- FASTING.....PP.....

RENAL FAILURE- GLOMERULAR FILTERATION RATE -.....SERUM CREATININE-.....

PREVIOUS HISTORY-.....

MENOPAUSE -

SYMPTOMS OF UTI -

DIABETES-(YES/ NO)-.....DURATION OF BEING DIABETIC-.....

INSULIN (iv)-

IMMUNOSUPPRESSIVE DISEASE (HIV OR ANY OTHER) -.....

HEALTH STATUS/HABIT -

QUESTIONNAIRE BY : SUSHILA PATHAK

मञ्जूरीनामा/CONSENT/ASSENT

म / मेरो यस रिसर्च “Prevalence of Urinary Tract Infection in Diabetic Patient and Assessment of Risk Factors of Urinary Tract Infection” मा मेरो सो इच्छाले भाग लिएको हुँ। यो विद्यार्थीहरूको एक शोध हो। यसमा म आफू राजीखुशीले निम्न सर्तमा यो मञ्जूरीनामामा सही छाप गरिदिएको छु। १ मेरो रगत/पिसाब यस अध्यनका लागि नमूनाका रूपमा दिएको छु। २ मेरो व्यक्तिगत कुरा गोप्य रहने छन। ३ म आफू राजीखुशीले कुनै पनि समयमा यो मञ्जूरीनामा फिर्ता लिन पाउने छु। ४ मैले सबै जानकारी तथा जोखिम बुझेर कसैको दबाव बिना आफ्नो सो इच्छाले यो मञ्जूरीनामामा मञ्जूरी जनाएको हुँ।

I/My..... have agreed to participate in this research on “Prevalence of Urinary Tract Infection in Diabetic Patient and Assessment of Risk Factors of Urinary Tract Infection” as a participant. I know this research is an academic process under the collaboration of National Kidney Centre, Banasthali and Central Department of Microbiology, Kirtipur. The procedures I agreed are the followings: 1. My urine or blood sample will be examined for this research. 2. My confidentiality will be maintained.3.My participation is voluntary. In any case I am free to withdraw my consent. 4. I have been explained and fully understand the risks involved and I have given this consent on my own will.

Name of participant (नाम)-

Address(ठेगाना) -

Date (मिति)-

Signature (सही)-

APPENDIX-II

Clinical profile of the patient

Urine Sample no.:Lab code no.:Date:
.....

Name of patient:
.....

Age:
.....

Sex:
.....Address.....

Day 1:

Time of sample collection:
.....

Sample- Urine	
Chemical Examination	Microscopic Examination
pH-	Pus cells- /Hpf
Albumin-	RBC- /Hpf
Sugar-	Epithelial cells- /Hpf
	Casts- /Hpf

Culture of specimen on: a) MA.....b) BA.....c)
SDA.....

Day 2:

1. Reading of culture plates

Colony characteristics on MacConkey agar/Nutrient agar / Sabouraud Dextrose agar

Shape.....
.....

Size.....
.....

Color.....
.....

Texture.....
.....

2. Different tests as required

Gram staining test:

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Catalase

test.....

Oxidase

test.....

Coagulase

test.....

Other biochemical tests

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Germ tube

test.....

The identified organism

was.....

.....

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3. Antibiotic Susceptibility Test

Antibiotic discs	Zone of inhibition (mm)	Result		
		Resistant	Intermediate	Sensitive
Gentamicin				
Cephalexin				
Cefotaxime				
Cotrimoxazole				
Cefoxitin				
Erythromycin				
Amikacin				
Imipenem				
Norfloxacin				
Nitrofurantoin				
Ofloxacin				
Nalidixic acid				

APPENDIX-III

Materials used during the course of study

Equipments used-

Autoclave:	Life +
Hot air oven:	Ambassador
Refrigerator:	Hitachi, Japan
Incubator:	Ambassador
Water bath:	Boekel 148003, Japan
Centrifuge:	Techno Fab
Weighing balance:	Choyo MP, Japan
Microscope:	Olympus, Japan
Test tubes/ Petriplates-	Borosil

Media used-

Nutrient agar,	MacConkey agar,
Blood agar,	Mueller Hinton agar,
Mueller Hinton broth,	Sabouraud Dextrose agar,
MR/VP medium,	Triple Sugar Iron agar,
Simmons Citrate agar	Sulfide Indole Motility agar.

The entire media used were from Hi Media Laboratories Pvt. Limited.

Chemical used-

Grams staining reagents,	Methyl red,
Oxidase,	Crystal violet,
Hydrogen peroxide,	Kovac's reagent,
Barritt's reagent,	Alpha-naphthalamine,
Hydrochloric acid and Phenol red.	

Test organisms-

E. coli (ATCC 25922), *S. aureus* (ATCC 25923), *K. pneumoniae* ATCC

Antibiotics used-

Gentamicin (10 μ g),
Cephalexin (30 μ g),
Cefotaxime (30 μ g),
Cotrimoxazole (25 μ g),

Norfloxacin (10µg),
Nalidixic acid (30µg),
Amikacin (15µg),
Nitrofurantoin (300µg),
Cefoxitin (30µg),
Ofloxacin (5µg),
Erythromycin (15µg) and
Imipenem (10µg).

These entire antibiotic discs used were from Hi Media Laboratories Pvt. Limited.

APPENDIX- IV

A. Composition and preparation of different media

1. MacConkey agar

Composition	Gm/lit
Peptic digest of animal tissue	17.0
Protease peptone	3.0
Lactose	10.0
Bile salt	1.5
Sodium chloride	5.0
Neutral red	0.03
Agar	15.0
Final pH at 25 °C	7.1±0.2

51.3 gram of the medium was dissolved in 1000 ml of distilled water and then boiled to dissolve completely. The media was autoclaved at 121°C (15 lbs pressure for 15 minutes. The sterilized medium was then poured into sterile petridishes and was allowed to cool.

2. Nutrient agar

Composition	Gm /lit
Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0
Agar	15.0
Final pH at 25°C	7.4±0.2

28 gram of the medium was dissolved in 1000 ml of distilled water and then boiled to dissolve completely. The media was autoclaved at 121°C (15 lbs pressure for 15 minutes. The sterilized medium was then poured into sterile petridishes and was allowed to cool.

3. Blood agar base (Infusion agar)

Composition	Gm/lit
Beef heart infusion form	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH at 25°C	7.3±0.2

42.5 gram of the medium was dissolved in 1000 ml of distilled water and then boiled to dissolve completely. The media was autoclaved at 121°C (15 lbs pressure for 15 minutes. After cooling to about 50-55°C, 5% v/v defibrinated sheep blood was added aseptically, then mixed with gentle rotation and poured into the sterilized petridishes and was allowed to cool.

4. Nutrient broth (NB)

Composition	Gm/lit
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH at 25°C	7.4±0.2

13.0 gram of the medium was dissolved in 1000ml of distilled water and then boiled to dissolve completely. The media was then dispensed into the tubes about 3ml in each and autoclaved at 121°C (15 lbs pressure for 15 minutes. The sterilized medium was then allowed to cool to room temperature.

5. Muller Hinton agar (MHA)

Composition	Gm/lit
Beef infusion	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH at 25°C	7.3±0.2

38gram of the medium was dissolved in 1000ml of distilled water and then boiled to dissolve completely. The media was autoclaved at 121° C (15 lbs pressure for 15 minutes. The sterilized medium was then poured into sterile petridishes and was allowed to cool.

B.Composition and preparation of different biochemical test media

1. Simon Citrate Agar

Composition	Gm/lit
Magnesium sulphate	0.2

Mono ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0
Final pH at 25°C	6.8±0.5

24.2 gram of the medium was dissolved in 1000 ml of distilled water and then boiled to dissolve completely. 3ml of media was then dispensed in each tube and autoclaved at 121°C (15 lbs pressure for 15 minutes). The sterilized medium was then allowed to settle at slant forming position.

2. Urea agar base (Christensen urea agar)

Composition	Gm/lit
Peptic digest of animal tissues	1.0
Dextrose	1.0
Monopotassium phosphate	0.8
Dipotassium phosphate	1.2
Sodium chloride	5.0
Agar	15.0
Phenol red	0.012
Final pH at 25°C	6.8±0.2

24.0 gram of the medium was dissolved in 950ml of distilled water and then boiled to dissolve completely and autoclaved at 121°C for 15 minutes. After cooling to 50°C, 50 ml of sterile 40% urea solution was poured into the medium and mixed with gentle rotation. Then 5 ml of the medium was dispensed in each tube and slant was prepared.

3. Sulphide indole motility (SIM) agar

Composition	Gm/litre
Peptic digest of animal	30.0
Beef extract	3.0
Peptonised iron	0.2
Sodium thiosulphate	0.025
Agar	3.0
Final pH at 25°C	7.3±0.2

36.23 gram of the medium was dissolved in 1000ml of distilled water and boiled to dissolve completely. Then it was dispensed in the test tube about 4ml and autoclaved at 121°C (15 lbs pressure for 15 minutes. The sterilized medium was allowed to cool down.

4. MR-VP medium

Composition	Gm/lit
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH at 25°C	6.9± 0.2

17.0 gram of the medium was dissolved in 1000ml of distilled water and boiled to dissolve completely. 3ml was dispensed in each test tube and autoclaved at 121°C (15 lbs pressure for 15 minutes.

5. Triple sugar iron (TSI) agar

Composition	Gm/lit
Peptic digest of animal tissue	10.0
Casein enzymatic hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate	0.2
Sodium thiosulphate	0.3
Agar	12.0
Phenol red	0.024
Final pH at 25°C	7.4± 0.2

65.0 gram of the medium was dissolved in 1000ml of distilled water and boiled to dissolve completely. Then it was dispensed into the test tubes and autoclaved at 121°C (15 lbs pressure) for 15 minutes. The sterilized medium was then allowed to set in slant with a butt of 1 inch thickness.

C. Composition and preparation of different staining reagent

1. Gram stain

a. Crystal Violet Solution

Crystal violet	20.0 gm
Ammonium oxalate	9.0 gm
Ethanol or Methanol	95.0 ml
Distilled water	1000ml

Preparation: 20 grams of crystal violet was weighed in a clean piece of paper and transferred to a clean brown bottle. Then 95.0 ml of ethanol was added and mixed until the dye is completely dissolved. To the mixture, 9.0 grams of ammonium oxalate dissolved in 200ml of distilled water was added. Finally the volume was made 1000ml by addition of distilled water.

b. Lugol's Iodine

Potassium iodide	20 gm
Iodine	10gm
Distilled water	1000ml

Preparation: To 250ml of distilled water, 20gm of potassium iodide was dissolved and 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1000ml by addition of distilled water.

c. Acetone Alcohol Decolorizer

Acetone	500ml
Ethanol (absolute)	475ml
Distilled water	25.0ml

Preparation: 475 ml of ethanol was added to 25ml of distilled water and mixed and kept in a clean bottle. Then immediately 500ml of acetone was added to the bottle and mixed well.

d. Safranin (Counter stain)

Safranin (2.5% in 95% ethanol)	10.0ml
Distilled water	100.0 ml

Preparation: 2.5% of safranin solution was prepared in 95% ethanol and 10ml of prepared suspension was mixed in 100ml of distilled water.

e. Normal saline

Sodium chloride	0.85gm
Distilled water	100.0 ml

Preparation : 0.85 gram of sodium chloride was weighed and added to a bottle containing 100ml of distilled water and mixed well to dissolve the salt completely and autoclaved. Then it was stored.

D. Biochemical test reagents and preparation procedures

a. Catalase Test

Catalase reagent (3% H₂O₂)

Hydrogen peroxide	1.0 ml
Distilled water	9.0 ml

Preparation: To the 9.0 ml of distilled water, 1ml of hydrogen peroxide was added and mixed well so as to make 3% solution of hydrogen peroxide.

b. Oxidase Test

Oxidase strip soaked in oxidase reagent

Tetra methylpara-phenylenediaminedihydrochloride (TPD)	1gm
Distilled water	100.0 ml

Preparation: 1 gram of TPD was dissolved in 100ml of distilled water and strips of Whatmann no.1 paper was soaked and drained for about 30 seconds. Then the strip was freeze and stored in dark bottle tightly.

c. Indole test

Kovac's indole reagent

Para dimethyl amino benzaldehyde	2.0 gm
Isoamyl alcohol	30.0 ml
Concentrated hydrochloric acid	10.0ml

Preparation: In 30ml of isoamyl alcohol, 2 gm of para amino benzaldehyde was dissolved and transferred to clean brown bottle. Then to this solution, 10ml of concentrated hydrochloric acid was added and mixed well.

d. Methyl red test

Methyl red solution

Methyl red	0.05gm
Ethyl alcohol	28.0ml

Distilled water 22.0ml

Preparation: 0.05 gm of methyl red was dissolved in 28ml of ethanol and transferred to a clean brown bottle. To this 22ml of distilled water was added and mixed well.

e. Voges Proskauer test

Barritt's reagent

Solution A

Alpha-Naphthol 5.0gm
Ethyl alcohol 100.0 ml

Preparation: 5 gm of α -Naphthol was dissolved in 25ml ethanol and transferred into clean bottle. Then final volume was made 100ml by adding ethanol.

Solution B

Potassium hydroxide (KOH) 40.0gm
Distilled water 100.0 ml

Preparation: 40 gm of KOH was dissolved in 25ml of distilled water and transferred into the clean bottle and final volume was made 100ml by adding distilled water.

f. Turbidity standard equivalent to McFarland 0.5

1% V/V solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of distilled water. 1% W/V solution of Barium Chloride was prepared by dissolving 0.5 gram of dehydrate barium chloride in 50 ml of distilled water. Then to the 99.5 ml of 1% sulphuric acid solution, 0.5 ml of barium chloride solution was mixed and stirred continuously. The solution was transferred into the clean screw capped tube and stored at dark place until used.

Morphology and Cultural characteristics of bacteria isolated from urine sample

Bacteria	Morphological characteristic	Cultural characteristics
<i>Escherichia coli</i>	Gram negative rod, 1-3 μ m x 0.4- 0.7 μ m, Aerobic and anaerobic, Nonsporing, Motile, noncapsulated	MA- Bright pink colonies due to lactose fermentation, smooth, glossy, translucent BA- Large 1-2 mm, grayish white, moist, smooth, convex and opaque. The colonies may appear

		mucoïd and some strains are haemolytic.
<i>Klebsiella spp.</i>	Gram negative, short, thick rods of 1-2 µm x 0.8 µm in size, nonsporing, nonmotile and capsulated.	MA and BA- Large dome shaped moist and usually viscous or mucoïd colonies
<i>Proteus spp.</i>	Gram negative rod, 1-3 µm x 0.4-0.6 µm, noncapsulated	MA- Non lactose fermenting colonies. BA- When cultured aerobically most strains are swarming type and have a fishy odour.
<i>Staphylococcus aureus</i>	Gram positive, Spherical cocci, 0.8-1.0 µm, Non sporing, facultative anaerobe, Non motile except for rare strains, non capsulated. They are arranged in characteristics grape like clusters or in small groups , pairs , singles and short chain (less than five cocci in line)	MA- Small, 0.1-0.5 mm, Pink or pink orange due to lactose fermentation. Some strains are non lactose fermenting. BA- Large 2-4 mm, Circular, smooth with glistening surface, entire edge, soft butyrous consistence and opaque and golden yellow to cream coloured pigmentation. Some strains are Beta haemolytic when grow aerobically.

E. ZONE SIZE INTERPRETATIVE CHART (CLSI interpretation)

Antibiotics	Disc content (µg)	Resistant (mm)	Intermediate(mm)	Sensitive(mm)
Amikacin	30	14	15-16	17
Cefotaxime	30	14	15-22	23
Cephalexin	30	14	15-17	18
Cotrimoxazole	25	10	11-15	16
Ofloxacin	5	12	13-15	16
Norfloxacin	10	12	13-16	17
Nalidixic acid	30	13	14-18	19
Nitrofurantoin	300	14	15-16	17
Cefoxitin	30	14	15-17	18
Imipenam	10	13	14-15	16
Erythromycin	15	13	14-22	23
Gentamicin	10	12	13-14	15

(Based on Results obtained using Mueller Hinton Agar)

Procedure for Gram staining procedure

Thin film of material to be examined was prepared and dried. The smear was heat fixed and allowed to cool before staining. The slide was flooded with crystal violet and allowed to remain without drying for 10 to 30 seconds. Then the slide was rinsed with tap water, shaking off excess. Then the slide was rinsed with iodine solution and allowed to remain on the solution without drying for 1 minute. The slide was then washed with tap water shaking off excess and flooded with acetone alcohol decolouriser for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolouriser. The slide was flooded with counter stain Safranin for 30 seconds and washed off with tap water. The slide was blotted between two sheets of bibulous paper and examined microscopically under oil immersion at 100X (Forbes *et al.* 2002).

Procedure for Germ tube test

A small inoculum made from isolated colony of yeast cell is suspended in 0.5ml of serum. The tube was incubated at 35° C for 3hours. A drop suspension was observed under microscope. Then the sporulating yeast cells observed indicated the germ tube test positive by *Candida albicans*.