# CHAPTER III

# MATERIALS AND METHODS

This study was conducted at the Queen Sirikit National Institute of Child Health, in Bangkok, from September to December 1998. The study protocol was approved by the ethical review committee of research committee, Ministry of Public Health, and hospital review committee.

## MATERIALS

### 1. TDx$^{R}$ Gentamicin

1.1 No.9512.01, Gentamicin Calibrators

Six vials with accurately measured amounts of gentamicin in normal human serum at following concentrations:

| Vial | Gentamicin concentration (μg/ml) |
|---|---|
| A | 0.0 |
| B | 0.5 |
| C | 1.5 |
| D | 3.0 |
| E | 6.0 |
| F | 10.0 |

Preservative: 0.1% Sodium azide

1.2 No. 9512.10, Gentamicin Controls

Three vials of gentamicin in normal human serum should read within the following range:

| Vial | Gentamicin concentration (μg/ml) |
|---|---|
| L | 0.85 –1.15 for 1 |
| M | 3.60 – 4.40 for 4 |
| H | 7.20 – 8.80 for 8 |

Preservative:0.1% Sodium azide

1.3 No. 9512.60, Gentamicin Reagent Pack :

The reagent pack consists of three vials:

| Vial | Component |
|---|---|
| P | Pretreatment solution: Surfactant in buffer containing protein stabilizer (3 ml) |
| S | Gentamicin antiserum (sheep) in buffer containing protein stabilizer (3ml) |
| T | Gentamicin – fluorescein tracer in buffer Containing surfactant and protein stabilizer (3ml) |

Preservative:0.1% Sodium azide

1.4 No. 9519.02, Buffer Dilution (For invitro diagnostic use)

The buffer solution was bovine gamma globulin in phosphate buffer.

Preservative: 0.1 sodium azide

**2. Apparatus**

2.1 Automated Fluorescence Polarization Analyzer ( Diagnostic Division, Abott Laboratories, Inc., Irving, TDx, U.S.A.)

2.2 Centrifuge (Clay adans, decton dickins, Inc., U.S.A (3400 rpm ))

2.3 Frezer (Ice Pack freezer, Model MRT 791, Electrolux, Inc., Sweden (-24 – - 30 °C)

## METHOD

**1. Subjects**

**Inclusion criteria**

Patients were enrolled in the study if they were treated with gentamicin either alone or with beta – lactam antibiotic due to suspected or proved bacterial infection. The subjects were infant age under 7 days, gestational age of 34 weeks or more, weight of at least 2,000 gm, and Apgar score of 4 or more at 1 minute, and 6 or more at 5 minutes.

**Exclusion criteria**

The patients were excluded if they were allergy to aminoglycoside, shock with renal failure, congenital malformations incompetent with life, anomalies of the kidney or ear, or presence of a neuromuscular disorder.

All of patient data related to the study were recorded such as gestation age, postnatal age, sex, weight, height, diagnosis, clinical status, vital sign, medical history, combination drugs, laboratory data such as serum creatinine, blood urea nitrogen, complete blood count (CBC), and culture sensitivity specimens. The data of patients were recorded by using patient medication profiles. (Appendix I).

**2. Dosage Regimen and Administration**

Neonatal patients in criteria were randomly assigned by the physicians to receive gentamicin either 2 – 2.5 mg / kg every 12 hours (TDD) or 4 – 5 mg / kg every 24 hours (ODD) by intravenous infusions at a constant rate for 30 minutes.

**3. Sample Collection**

3.1 Blood samples were collected and several laboratory data were monitored.

- Serum creatinine, and blood urea nitrogen were determined before gentamicin therapy, every 3 days during therapy, and after gentamicin therapy.
- Complete blood count (CBC), were determined before gentamicin therapy ,and immediately after gentamicin therapy.
- Body temperature, was monitored every day until gentamicin therapy was terminated.

3.2 The blood samples (1.0-1.5 ml)of patients receiving either dosage regimen were collected to assay for gentamicin serum concentration when the blood levels were considered to be at steady – state

3.2.1 Patients receiving gentamicin 2 – 2.5 mg/kg every 12 hours.

Sample 1: The trough blood sample was drawn within 30 minutes prior to the $5^{th}$ does .

Sample 2: The peak blood samples of gentamicin therapy was drawn within 30 minutes after the completion of 30 minutes intravenous infusion of the $5^{th}$ doses.

3.2.2 Patients receiving gentamicin 4 – 5 mg / kg every 24 hours.

Sample 1: The trough blood sample was drawn within 30 minutes prior to the 3rd dose .

Sample 2: The peak blood sample was drawn within 30 minutes after completion of the intravenous infusion of the 3rd dose.

As soon as clotting had occurred serum was separated by centrifugation (3,400 rpm for 10–15 minutes) at room temperature, and they were analyzed by Fluorescence Polarization Immunoassay Technology (TDx® Analyzer system , Abbott). If the samples were not immediately analyzed, they were kept frozen at - 24 to -30° C in the freezer until assayed within 24-48 hours.

Adjustment of gentamicin dosage regimen

Adjustment of gentamicin dosage or dosing interval were recommended in an attempt to provide maximal potential benefit with minimal risk of toxicity if the gentamicin concentration was not in the therapeutic range.

- Patient receiving gentamicin 2 – 2.5 mg / kg every 12 hours.

Adjustments were recommended for $C_{pk}$ which was less than 4 mg/l or higher than 12 mg/l, and $C_{tr}$ which was higher than 2 mg/l .[14,19]

- Patient receiving gentamicin 4 – 5 mg / kg every 24 hours.

Adjustments were recommended for $C_{pk}$ which was less than 5 mg/l or higher than 18 mg/l, or $C_{tr}$ which was higher than 1.5 mg/l .[33]

## 4. Analytical Method

Gentamicin levels in serum samples were determined by immunoassay method using TDx[R] Analyzer system, Abbott Laboratories based on fluorescence polarization technique.

### 4.1 Perform an assay calibration

The equipments consisted of calibration carousel, cuvettes, sample cartridges, reagent pack, calibrators and controls. The calibration method was done as follow:

1. Prepared the carousel by loaded the carousel with 15 cuvettes and cartridges in positions no.1 to no.15.
2. Mixed calibrators A to F approximately 3-5 times and pipetted at least 50 µl of calibrators into the 1 st to the 12 th sample wells.
3. Mixed controls L to H approximately 3-5 times and pipetted at least 50 µl of control into the 13 th to the 15 th sample wells.
4. Mixed the reagents approximately 3-5 times, opened the covers of the reagents, and loaded the reagent pack in the instrument.
5. Loaded the carousel in the instrument.
6. Closed the door.
7. Pressed run.
8. The instrument commenced operation ,and waited for run to complete.
9. Kept the printout for later discussion.

* Remark: Made sure that the air bubbles were not happened when the calibrator, controls and reagents were mixed.

An acceptable gentamicin calibration curve should meet the following criteria:

1. Polarization error (PERR) or percent error values was less than or equal 3.5.
2. The root mean square error (RMSE) was less than or equal to 2.0.
3. All controls were within the acceptable ranges.

**4.2 Performing an Assay Run.**

The equipments consisted of assay carousel, sample cartridges, and reagent pack. The method for performing assay run was done as follow:

1. Prepared the carousel by loaded the carousel with 3 cuvettes and cartridges in positions no.1 to no.3.(for 3 specimens)
2. Pipetted at least 50 µl of serum sample into the 1 $^{st}$ to the 3 $^{rd}$ sample wells. (specimen # 1 in well # 1, specimen # 2 in well #2, and specimen # 3 in well # 3)
3. Mixed the reagents approximately 3-5 times, opened the covers of the reagents, and loaded the reagent pack in the instrument.
4. Loaded the carousel in the instrument.
5. Closed the door.
6. Pressed run.
7. The instrument commenced operation, and waited for run to complete.
8. Kept the printout for later discussion.

**5. Data analysis**

**5.1 Characteristics of patients**

- Percentage and chi – square test (Yates' Correction) were used for comparison of sex between the two groups.

- Gestational age, postnatal age, weight, height, Apgar score and duration of treatment between the two groups were compared using unpaired t-test.
- Demonstrated the percentage of disease indicated gentamicin treatment, concomitant antibiotic, and other diseases of the patients in both groups.

**5.2 Gentamicin peak and trough levels.**

- The mean steady state peak and trough gentamicin levels between the two groups were compared using unpaired -t test.
- Demonstrate the percentage of the patients in the TDD and ODD groups whose peak level was in different range that was < 5 mg/l, < 8 mg/l, ≥ 8 mg/l, and >12 mg/l.
- Demonstrate the percentage of the patients who had subtherapeutic peak (less than 4 mg/l) and/or too high trough gentamicin serum concentration was too high (more than 2 mg/l In the TDD group and more than 1.5 mg/l in the ODD group).

**5.3 Pharmacokinetic parameter**

Pharmacokinetic parameter such as elimination rate constance, volume of distribution, elimination half life, clearance of gentamicin were calculated by using equation 5.3.1-5.3.4.

- Comparison of the mean elimination rate constant (K), elimination half-life ($t_{1/2}$), volume of distribution (Vd) and clearance of gentamicin (Cl) by unpaired – t test.
- Demonstrate the correlation between clearance of gentamicin in l/hr and serum creatinine concentration in mg/dl or creatinine clearance in ml/min/1.73 $m^2$ or creatinine clearance in l/hr by linear regression analysis.
- Demonstrate the correlation between clearance of gentamicin in l/hr and posnatal age in day, weight in gram, and serum creatinine concentration in mg/dl or creatinine clearance in ml/min/1.73 $m^2$ or creatinine clearance in

l/hr by multiple linear regression.

Equations

5.3.1 $$K = \frac{\ln ( C_{pk} / C_{tr} )}{\Delta t} \quad \text{hour -1}$$

**K** = Elimination rate constant

**Cpk** = Peak concentration

**Ctr** = Trough concentration

$\Delta$ **t** = Time interval between the peak and trough concentrations

5.3.2 $$t1/2 = 0.693/K \quad \text{hour}$$

$t_{½}$ = Elimination half – life

5.3.3 Use Bolus model because the elimination half- life time more than 6 times of infusion time.

$$Vd = \frac{SFD \times e^{-Kt}}{Cp (1 - e^{-KT})}$$

**Vd** = volume of distribution

**S** = salt form

**F** = bioavailability

**D** = dose

$C_p$ = the measured plasma concentration

**t** = time interval between the start of the infusion and the time at which the measured plasma concentration.

**T** = dosing interval

5.3.4 **Cl = K( Vd )**

**Cl** = Clearance of gentamicin

**5.4 Renal parameter**

Baseline and after treatment serum creatinine data and creatinine clearance (calculated by equation 5.4.1) of the patients in both gentamicin dosage regimen groups were compared within group and between groups. Toxicity was indicated if the serum creatinine of the patient with normal renal function increased 0.5 mg/dl or more from the baseline, or when the increment was 50% of the normal value .

- Comparison of the mean of serum creatinine concentration at the first day, third day, and discontinuous day within TDD group and ODD group by paired- t test.
- Comparison of the mean of serum creatinine concentration at the first day between the TDD and the ODD group by unpaired – t test .
- Comparison of the mean of serum creatinine concentration at the third day, and discontinuous day between the TDD and the ODD group by ANCOVA analysis.
- Comparison of the mean of serum creatinine concentration between the third day and the discontinuous day that were divided to different period such as 4 -5 days, 6 – 8 days, $\geq$ 9 days by paired – t test.
- Demonstrate the correlation between the duration of gentamicin therapy and the changing of serum creatinine level at discontinuous day from the third day of the patients in the TDD and the ODD groups.

Equation (Schwartz, Brion, and Spitzer, 1987 )[42]

5.4.1 **CLcr = K x L / S cr**

**CLcr** = Creatinine clearance ml / min / 1.7.3 $m^2$

**K** = Constant of proportionality that is age specific

| | Age | K |
|---|---|---|
| Low birth weight (< 2500 mg ) | ≤ 1y | 0.33 |
| Full term | ≤ 1y | 0.45 |

**L** = Length in cm

**Scr** = Serum creatinine concentration mg/dl

**TABLE 3.1 Serum creatinine level in infants**[71]

| Age | Serum creatinine (mg/l) |
|---|---|
| 1 day | 0.80-1.00 |
| 2 days | 0.70-0.80 |
| 3 days | 0.70-0.80 |
| 5 days | 0.70 |
| 7 days | 0.60 |
| 1-2 weeks | 0.34-0.66 |
| 2-26 weeks | 0.23-0.55 |

**5.4 Drug interaction of gentamicin**

- Demonstrate the percentage of the patients in the TDD and the ODD groups who was given the drug that interact with gentamicin.

**5.6. Efficacy**

- Comparison steady state gentamicin peak level, duration, and efficacy response of the patients with different indications for gentamicin between the TDD and the ODD groups by unpaired -t test.

Evaluation of Efficacy

5.6.1 Clinical efficacy

Favorable clinical response

When the patients' symptoms was clinically improved, or normalized, the symptoms were defined as favorable. Normal clinical signs and symptoms were defined when the patients had normal feeding, normal activity, normal response, normal crying, and normal Moro' s rflex (normal flexion of extremities), normal respiratory rate (40 – 60 breaths per minute), had no respiratory difficulty and apnea etc.

Unfavorable clinical response

When the patients' symptoms was not clinically improved with resolution of infection symptoms, it was indicated as unfavorable clinical response.

5.6.2 Some laboratory data used in combination to determine clinical efficacy

5.6.2.1 Microorganism culture

Favorable microorganism culture

- Negative culture
- New microorganism, no signs of infection

Unfavorable microorganism culture

- Culture still positive
- No new microorganism, signs of infection

Not evaluable

- No positive culture at the start of therapy

5.6.2.2 Temperature

Favorable

- Return to normal temperature (36.0 – 37.8$^{O}$C)[72–73] within 3 days after therapy.

Unfavorable

- Temperature higher than 37.8$^{O}$C or lower than 36.0 $^{O}$C

Not evaluable

- Normal temperature at start therapy

5.6.2.3 While blood cell count

Favorable

- Normalization while blood cell count ($5.0 – 25.0 \times 10^{3}$ /mm$^{3}$)

Unfavorable

- Abnormality of while blood cell count (WBC)

Not evaluable

- Normal white blood cell count at start therapy.

Patients were define as favorable by physician, when the results in 5.6.1 or clinical responses was favorable along with the laboratory support in 5.6.2 was

favorable or was not evaluable. Patients were defined as unfavorable. when the results in 5.6.1 unfavorable along with the results in 5.6.2 which was also unfavorable, this could occurred when the patients got infection.