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Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A (2003). TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. J. Biomed. Res. 66:476-82.

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Research Articles

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Full Length Research Paper

The effect of insulin therapy and plasma glucose levels on corrected QT intervals in patients with type 2 diabetes

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Accepted 24 August, 2011

This study aims is to investigate the effect of medium-term insulin therapy (3 months) and plasma glucose levels on corrected QT intervals (QTc) in patients with type 2 diabetes. The subjects were 17 patients with type 2 diabetes who had poor glycemic control and were changed to insulin therapy from sulfonylurea or diet therapy alone. QTc, fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), serum potassium and body weight were measured at baseline and after 3 months of insulin therapy. A significant increase of QTc (0.406 ± 0.027 to 0.421 ± 0.025 s, P =0.0025) and a significant decrease in HbA1c (10.9 ± 1.9 to 7.9 ± 1.7%, P =0.0002) were found after 3 months of insulin therapy. QTc showed no correlation with FPG, HbA1c or body weight before insulin therapy and the change in QTc was not correlated with the change in FPG, HbA1c or body weight from before to after insulin therapy. In this study, insulin therapy for 3 months caused a significant increase of QTc in patients with type 2 diabetes. However, given the small size of the trial, further studies with larger number of patients are needed.

Key words: QTc, type 2 diabetes, insulin therapy.

INTRODUCTION

The corrected QT intervals (QTc) on an electrocardiogram (ECG) are prolonged in patients with diabetes complicated by diabetic autonomic neuropathy (Kahn et al., 1987; Chambers et al., 1990) and the prolongation is associated with mortality in such patients (Sawicki et al., 1996). QTc prolongation may also be related to circulating insulin levels and insulin resistance (Dekker et al., 1996; Festa et al., 1999; Kazumi et al., 1999) or obesity (El-Gamal et al., 1995). A recent report also demonstrated that experimental acute insulin therapy can prolong QTc (Gastaldelli et al., 2000). In contrast, it has also been reported that acute hyperglycemia produced by the glucose clamp method can induce QTc prolongation in healthy subjects and patients with type 1 diabetes (Gan et al., 2009). However, it is unclear how improvement of glycemic control by newly initiated insulin therapy influences QTc over a period of a few months, although one study showed that insulin therapy reduced QTc at 4-month follow-up in patients with type 2 diabetes (Schnell et al., 2004). Main purpose of this study is to investigate the effect of insulin therapy and plasma glucose levels on QTc in patients with type 2 diabetes. Our study showed that insulin therapy for 3 months caused a significant increase of QTc in patients with type 2 diabetes, despite the improvement of glycemic control.

PATIENTS AND METHODS

Patients

The effect of newly initiated insulin therapy on QTc was investigated in 17 patients with type 2 diabetes (9 men and 8 women) who had poor glycemic control and were changed to insulin therapy from sulfonylurea or diet therapy alone. There was no washout period of

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Abbreviations: QTc, corrected QT interval, **FPG,** fasting plasma glucose, **HbA1c,** hemoglobin A1c.

Table 1. Clinical characteristics of the non-diabetic and diabetic Japanese subjects.

Characteristics	Non-diabetes	Diabetes (Before insulin)	Diabetes (After insulin)	P	P[a]
No. (male/female)	30 (14/16)	17 (9/8)	—	—	—
Age (year)	60.2 ± 4.9	58.4 ± 10.6	—	0.9128	—
Duration of diabetes (year)	—	13.1 ± 8.7	—	—	—
QTc (s)	0.388 ± 0.026	0.406 ± 0.027	0.421 ± 0.025	0.0327*	0.0025*
FPG (mg/dL)	90.1 ± 6.81	83.0 ± 49.8	149.9 ± 61.6	0.0001*	0.1598
$HbA1_C$ (%)	4.9 ± 0.3	10.9 ± 1.9	7.9 ± 1.7	0.0001*	0.0002*
BMI (kg/m^2)	21.7 ± 1.9	23.6 ± 5.1	—	0.5348	—
Body weight (kg)	—	61.6 ± 16.2	62.8 ± 16.8	—	0.1391
Diabetic therapy					
SU	—	12	—	—	—
(SU1/SU2/SU3/SU4)	—	1/3/3/5	—	—	—
Diet alone	—	5	—	—	—
Antihypertensive drugs	—	7	—	—	—
(Ca /Ca+ARB)	—	5/2	—	—	—

Data are expressed as mean ± standard deviation (SD). Comparison in variables between two groups was made by use of an unpaired *t* test. P: p value of Non-diabetes vs. Diabetes (before insulin), P[a]: p value of Diabetes (before insulin) vs. Diabetes (after insulin), P <0.05 are defined as statistical significance (*). FPG: fasting plasma glucose, $HbA1_C$: hemoglobin $A1_C$, BMI: body mass index; Insulin: insulin therapy, SU: sulfonylurea, α-GI: α glucosidase inhibitor; SU1:SU alone, SU2: SU and α-GI, SU3: SU and metformin, SU4: SU, metformin and α-GI; Antihypertensive drugs: Ca: calcium channel blocker, ARB: angiotensin-II receptor blocker.

sulfonylurea before initiating insulin therapy. These patients were prospectively and consecutively enrolled in the study from October 2005 to February 2006. The clinical characteristics of the patients are summarized in Table 1. Patients with evidence of liver dysfunction, infectious disease or autoimmune disease, autonomic disorder other than diabetes mellitus, hypothyroidism, renal failure, and cardiac failure were excluded from the study. Patients treated with drugs which may influence QTc such as β blockers, anti-arrythmic drugs, and diuretics were also excluded. No patients showed apparent ketoacidosis evaluated by urine tests. QTc measurements were also performed in 30 healthy controls.

METHODS

QTc measurement: An ECG for QT measurement was recorded in the morning before breakfast after a 10 h overnight fast on the same day that blood and urine tests were performed. During the recording, the subjects were at rest in a supine position and were instructed to maintain a respiratory rate of >9 breaths/min to decrease the effect of respiratory sinus arrhythmia. No patient had an increased QRS duration. The QT interval was measured automatically using an ECG instrument FX-7412 (Fukuda Denshi Limited, Tokyo, Japan). In previous studies (Takebayashi et al., 2002; 2004), we have measured QTc manually, but technical difficulties with this approach caused us to use the automatic method in the current study. The QT interval was determined as the mean of 12 leads for each beat and QTc was calculated using Bazett's formula: $QTc = QT / RR^{1/2}$ (Bazett, 1920). The QTc for each patient was defined as the mean QTc over 100 consecutive beats. To prevent over- or underestimation of the QT interval by Bazett's formula (Puddu et al., 1988), only individuals with a normal pulse rate (60 to 100 beats/min) in resting state were enrolled in the study.

Blood tests

After collection, the blood was rapidly centrifuged at 1500 rpm for 5 min to separate serum and plasma from clot-containing blood cells. These samples were stored at -70°C until analysis. Plasma glucose, HbA_{1C}, and serum lipid concentrations were measured as described previously (Takebayashi et al., 2004). Serum potassium concentration was measured by the dilution method by ion selective equipment using automated analyzer of electrolyte (JCA-BM2256, JOEL Ltd., Tokyo, Japan).

Insulin therapy

Insulin therapy was initiated at a dose equal to approximately the body weight of the patient (kg) × 0.2 units, and then the dose was adjusted based on the degree of glycemic control. Blood tests were performed at the start of insulin therapy and after 12 weeks; in each case before breakfast after at least a 10 h period of overnight fasting. No change in administration of any drug occurred for any patient during the 12-week study period.

Regarding antihypertensive drugs, 5 patients received amlodipine (5 mg/day) and 2 patients received amlodipine (5 mg/day) and olmesartan (20 mg/day). Patients who received other anti-hypertensive drugs were not included in this study.

In this study, QTc prolongation was defined as "prolongation" from baseline QTc (= before initiation of insulin therapy) even if the

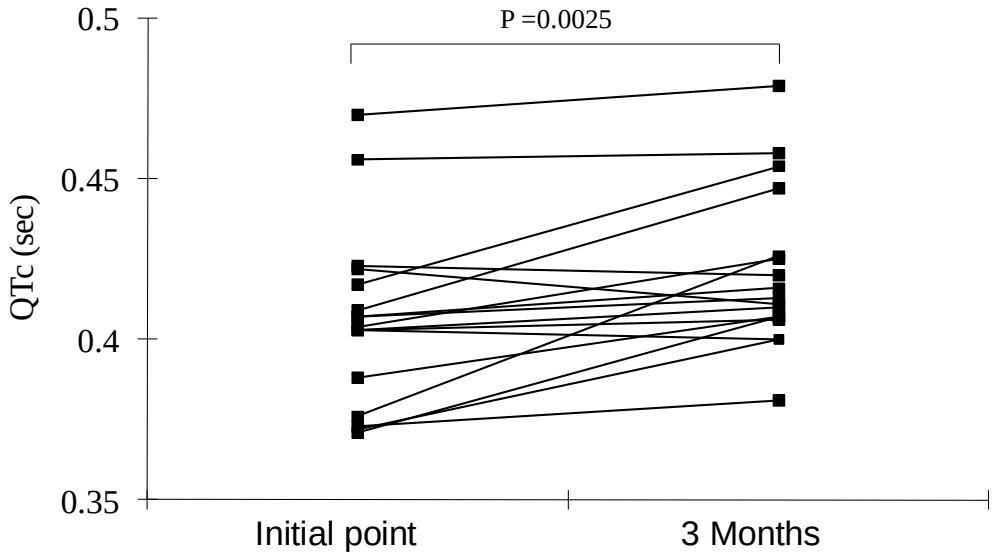

Figure 1. Effect of 3 months-insulin therapy on corrected QT intervals in patients with type 2 diabetes.

QTc after insulin therapy was still within normal range (QTc ≦ 0.43 s in men and ≦ 0.45 s in women) defined by the most recent European regulatory guidelines (Straus et al., 2006).

Ethical considerations

*All subjects gave informed consent to inclusion in the study, which was performed according to the guidelines in the declaration of*Helsinki.

Statistical methods

All data are presented as means ± standard deviation (SD). The significance of a correlation between two variables was determined by simple regression analysis. Comparison of two time points for an individual was performed using a paired t test. A P value of less than 0.05 was considered to indicate statistical significance in all analyses.

RESULTS

The mean QTc values in the 30 healthy controls and the mean baseline QTc values in the 17 patients with type 2 diabetes were 0.398 ± 0.026 and 0.406 ± 0.027 s, respectively, with no significant difference between these groups (P =0.3277). The QTc showed significant prolongation to 0.421 ± 0.025 s (P =0.0025) after 3 months of insulin therapy in the patients (Figure 1), and there was a significant difference in the mean QTc after treatment compared to the QTc in the healthy controls (P =0.0052).

A tendency for a decrease in FPG (183.0 ± 49.8 to 149.9 ± 61.6 mg/dL, P =0.1598) and a significant decrease in $HbA1_C$ (10.9 ± 1.9 to 7.9 ± 1.7%, P =0.0002) were also detected after the 3 months of insulin therapy. There were no significant changes in body weight (61.6 ± 16.2 to 62.8 ± 16.8 kg, P =0.1391), potassium (4.2 ± 0.4 to 4.1 ± 0.4 mEq/L, P =0.2342), SBP (130.4 ± 11.1 to 126.2 ± 14.1 mmHg, P =0.2979) or DBP (74.1 ± 12.3 to 71.3 ± 7.7 mmHg, P =0.3335) after insulin therapy.

There was no correlation of QTc with FPG (R =0.3521, P =0.1657), HbA1c (R =0.0457, P =0.8619) or body weight (R =0.0723, P =0.7827) at the start of insulin therapy. Similarly, the change in QTc from before to after insulin therapy showed no correlation with the change in FPG (R =-0.2432, P =0.3469), $HbA1_C$ (R =-0.1856, P =0.4686) or body weight (R =0.1309, P =0.6166) from before to after therapy.

There was also no correlation between QTc and the serum potassium concentration at the start of insulin therapy (R =0.1656, P =0.5558). However, a tendency for a negative correlation was observed between the changes in QTc and serum potassium from before to after insulin therapy (R =-0.3753, P =0.1377).

Mean insulin dose after the 3 months of insulin therapy was 19.8 ± 10.0 U/day. There were no patients showing polymorphic ventricular fibrillation on ECG after the 3 month of insulin therapy.

DISCUSSION

In the current study, we investigated the medium-term effect of insulin therapy for 3 months on QTc in patients with type 2 diabetes. Insulin therapy significantly increased QTc compared with that at baseline, and the QTc after therapy was significantly longer than that in healthy subjects. The QTc in the diabetic patients at baseline did not differ significantly compared with the QTc

in healthy subjects. We speculate that this was due to the small number of patients in this study, since we have previously shown significant QTc prolongation in diabetic patients compared with QTc in healthy subjects (Takebayashi et al., 2004), supporting previous study (Borra and Gea, 2001).

It has been shown that experimental acute insulin therapy prolongs QTc (Gastaldelli et al., 2000) and we have previously found significant QTc prolongation in patients with type 2 diabetes patients treated with insulin therapy compared with those treated with dietary modification alone in a cross-sectional study (Takebayashi et al., 2002). In addition, Sakabe et al. reported that among type 2 diabetic patients with post myocardial infarction treated with insulin, sulfonylurea, or diet alone, corrected QT dispersion was significantly greater in the insulin group (Sakabe et al., 2008). A significant positive correlation between QTc and plasma insulin levels has also been shown (Kazumi et al., 1999). Our current results appear to support these findings. In contrast, it is also reported that acute hyperglycemia can cause QTc prolongation (Gan et al., 2009), although there are reports showing that hypoglycemia also causes QTc prolongation (Christensen et al., 2010 a, b). Importantly, QTc was prolonged in the current study despite the large reduction in HbA1c (by approximately 3%) after 3 months of insulin therapy. Furthermore, QTc was not correlated with FPG or HbA1c at baseline and the changes in these parameters from before to after insulin therapy also showed no significant correlation. This appears to show that plasma glucose level did not cause prolonged QTc. Therefore, the role of glucose level in the clinical pathogenesis of prolonged QTc was not fully evident at least in this study. Taken together, we speculate that medium-term (but not acute) insulin therapy prolongs QTc independently of glycemic control.

Interestingly, in the recently published ACCORD study (Gerstein et al., 2008), patients who received intensive glucose lowering therapy (a higher percentage of insulin therapy and a higher dose of insulin) had greater mortality than those who received conventional therapy. The increased mortality with intensive therapy appeared to be explained by the higher incidence of severe hypoglycemia in these patients. However, it may be of interest to examine the influence of intensive insulin therapy on QTc, because QTc prolongation is associated with mortality in patients with diabetes (Sawichki et al., 1996).

The detailed mechanism of induction of QTc prolongation by insulin is unknown, but an influence of the serum potassium concentration on acute insulin effects has been suggested (Gastaldelli et al., 2000). In this study, there was no correlation between QTc and serum potassium at start of insulin therapy. We speculate that this negative result was due to small number of patients at least partially. However, we found a tendency for a negative correlation between the changes in QTc and serum potassium levels from before to after insulin therapy, in support of this proposed mechanism (Gastaldelli et al., 2000). However, this association was not always strong and other mechanisms may also be involved such as the change of other ions (including calcium ion) concentrations. Furthermore, ion channel gene SNPs in individual patients may have influenced QTc as reported by Gouas et al. (2007).

In the current study, the BMI of this study, which may influence QTc (El-Gamal et al., 1995), was not significantly increased compared to control group. This may be due to the fact that patients in this study were all Japanese. It is reported that Japanese type 2 diabetic patients' BMI are almost 23 kg/m^2 in large number of study (Sone et al., 2002), which corresponded to those in this study.

The study has some limitations. First, the number of patients was small and we did not perform power calculation for sample size before initiating the study. In addition, we did not use a control group of diabetic patients for comparison with the insulin-treated patients. It would have been interesting to investigate the effects on QTc of treatment with thiazolidinediones or metformin, which can decrease circulating insulin levels while decreasing plasma glucose based on improved insulin resistance. Second, administration of sulfonylureas was suspended after initiation of insulin therapy, but we were unable to use washout periods for these drugs for ethical reasons. Third, according to the most recent European regulatory guidelines (Straus et al., 2006), QTc $\leqq$0.43 s and $\leqq$0.45 s in men and women is respectively considered as normal. Therefore, the QTc in this study was still within normal range even after the insulin therapy. Because the patients in this study at baseline had relatively low QTc, it may also be important to evaluate the effect of insulin therapy on QTc in patients' population with higher QTc (borderline QTc; that is 0.450 $\geqq$QTc $\geqq$0.431 s in men and 0.470$\geqq$QTc $\geqq$0.451 s in women). Forth, it would have been interesting to see and compare the effect of initiation of insulin therapy on QTc not in only type 2 diabetes but also in type 1 diabetes. In addition, it would have been important to compare the effect of insulin treatment duration on QTc. Furthermore, plasma insulin levels should have been measured. Finally, because it is well documental fact that hypocalcemia prolongs QTc, serum calcium levels should have been measured to assess the association between these levels and insulin therapy. Within these limitations, we conclude that 3-month insulin therapy causes a significant QTc prolongation in patients with type 2 diabetes. A more detailed analysis is required to establish the basis of the effect of insulin on QTc.

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Full Length Research Paper

Calculated free testosterone in hemodialysis and renal transplanted men: Comparison of three equations with each other and with free androgen index

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Free testosterone has been shown to correlate better with androgen status than total testosterone. Assessment of free testosterone level has been recommended by all consensus best practice guidelines; however, its direct measurement is laborious and out of the scope of most clinical service laboratories. Calculating its level using some equations has been advocated in the absence of a direct measuring method. Androgen deficiency has been shown to be more prevalent in hemodialysis and renal transplanted patients compared to normal individuals. This study aimed to calculate free testosterone level in those patients using different equations to test whether these equations correlate with each other and with free androgen index. A significant discrepancy was found when these equations were compared with each other and when compared with free androgen index. In conclusion, free testosterone equations gave significantly discordant results when applied to male hemodialysis and renal transplanted patients. Therefore, we recommended validating these equations by free testosterone reference method before applying them to such patients.

Key words: Free androgen index, free testosterone, hemodialysis, transplantation.

INTRODUCTION

In men, testosterone is the most important and abundant androgen in blood (Saez, 1994). Its deficiency has detrimental consequences on many organ systems (Greenspan et al., 1986; Jockenhovel et al., 1997; Davidson et al., 1979; Kapoor et al., 2005; Després et al., 1996).

Endocrine abnormalities are a common feature of chronic renal insufficiency (Schaefer et al., 1992; Clodi et al., 1998). Changes of testosterone synthesis and metabolism develop early after the onset of renal insufficiency. To avoid the consequences of hypogonadism in these patients a reliable index of androgenic status should be used.

Serum total testosterone may not always reflect the exact androgen status of a subject. Testosterone bound to SHBG is considered as biologically inactive (Anderson, 1974); therefore an estimate of the non-SHBG-bound fraction is considered a more reliable measure of androgen status. Free testosterone measurement is recommended as part of clinical evaluation for androgen deficiency by all consensus best practice guidelines (Bhasin et al., 2010; Petak et al., 2002; Nieschlag et al., 2005; Conway et al., 2000). The reference method for estimating free testosterone is equilibrium dialysis (Sinha-Hikim et al., 1998) or centrifugal ultrafiltration (Hammond

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et al., 1980).

These methods are laborious and not suitable for a routine clinical service. Therefore, clinicians had to rely simply on the total testosterone or to use calculated indices of biologically available testosterone such as free androgen index (FAI) (FAI = total testosterone X 100/SHBG) (Wilke and Utley, 1987) or more complicated calculations to derive free testosterone concentration (Vermeulen et al., 1999; Sodergard et al., 1982), which is more practical alternative and has lately been advocated (Diver, 2006).

The performance of free-testosterone calculating equations in healthy subjects has already been evaluated. Up to our knowledge, this is the first study that investigates the performance of those those equations in diseased subjects known to have endocrine disturbance that encompasses impaired testosterone production and metabolism.

The current study aimed at calculating free testosterone level by three published equations in hemodialysis, renal transplanted patients, and age matched healthy individuals and to test whether they correlated with each other and with FAI.

PATIENTS AND METHODS

Subjects

Morning blood samples were withdrawn from 49 hemodialysis patients (pre-dialysis), 44 renal transplanted patients, and 63 age-matched controls after obtaining ethical approval and informed written consents. Hemodialysis patients underwent regular polysulfone lowflux dialysis (Hemoflow F 7 HPS, Fresenius AG) three times a week (4 h/per session). Renal transplanted patients were on regular immunosuppressive medications: prednisolone from 5 to 10 mg/day, mycophenolate mofetil from 1 to 2 g/day, and cyclosporine A (trough level 129 ± 38.5 ng/ml) or tacrolimus (trough level 6.3 ± 1.9 ng/ml), according to the donor/recipient compatibility. The inclusion criteria for all participants were: male gender and age between 20-50 years. For hemodialysis patients, only those who have been on 3 times per week dialysis for more than 6 months have been included. Only renal transplanted patients with ≥ 6 months well-functioning graft (glomerular filtration rate > 60 ml per minute were included. We excluded any subject on testosterone replacement therapy, had diabetes mellitus, had history of endocrinological disorder, or liver disease. Any renal transplanted patient with history of graft rejection (that is, had features of rejection in graft biopsy, based on Banff criteria) was also excluded.

Biochemical investigations

Serum samples were assayed for urea; creatinine, sodium, potassium, glucose, and albumin were assayed by RxL Chemistry Analyzer (Dade Behring Inc, Newark, DE). SHBG and serum total testosterone concentrations were measured by Architect i2000 analyzer (Abbott Laboratories, Chicago, IL) that employed Chemiluminescent Microparticle Immunoassay method (CMIA). The assay lower detection limit was 0.28 and 0.36 nmol/L for testosterone and SHBG; respectively. Total testosterone assay had 4.5 and 1.9% within-assay coefficients of variation (CV) at total testosterone concentrations of 2.84 and 17.5 nmol/L; respectively. The between-assay CV for total testosterone were 8.0 and 3.7% at total testosterone concentrations of 2.84 and 17.5 nmol/L; respectively. SHBG assay had 4.78 and 4.8% within-assay coefficients of variation (CV) at SHBG concentrations of 8.8 and 24.5 nmol/L; respectively. The total CV for SHBG were 9.5 and 5.7% at SHBG concentrations of 8.8 and 24.5 nmol/L; respectively. The other analytes

Free testosterone calculation

Three equations to calculate free testosterone (CFT) were compared in this study:

(1) Vermeulen et al. (1999) equation (CFTV):

$$CFTV = [T - N - S + \sqrt{((N + S - T)^2 + 4NT)}] / 2N$$

Where T, S and A are total testosterone (nmol/L), SHBG (nmol/L) and albumin (g/L) concentrations, respectively, and N = 0.5217 A + 1.

They based their calculations on the law of mass action and verified the obtained free testosterone results by equilibrium dialysis measurement. The equation for calculated free testosterone was not reported in the original study, however, Ho et al. (2006) used the association constants of testosterone for SHBG (10^9 L/mol) and albumin (3.6×10^4 L/mol) quoted by the authors and derived the aforementioned equation.

(2) The Nanjee-Wheeler equation (CFTNW) which they derived from regression analysis of free testosterone concentrations measured by a gel filtration method in serum samples from 100 normal men and 18 normal women (Nanjee and Wheeler, 1985). Statistical treatment of the original data took into account the changes of testosterone and SHBG but not albumin concentration and yielded the following equation:

$$CFTNW = T \times (6.11 - 2.38 \times \log_{10} S)$$

where T and S are total testosterone and SHBG concentrations, respectively (nmol/L).

(3) More recently, Sartorius et al. (2009) developed an additional novel empirical formula (CFTZ):

$$CFTZ = 24.00314 \times T/\log_{10} S - 0.04599 \times T^2$$

Where S: is SHBG in nmol/L and T: is total testosterone in nmol/L. In the same study, they evaluated the predictive accuracy of CFTZ and other four equations including CFTV and CFTNW using a database of nearly 4000 consecutive blood samples from a routine diagnostic laboratory. Free testosterone was measured by centrifugal ultra-filtration, a centrifuge-accelerated form of equilibrium dialysis (Vlahos et al., 1982). Total testosterone and SHBG levels were measured by immunoassays on every sample included in that study. They concluded that CFTZ had a high predictive accuracy in both model fit and predictive error estimation. CFTZ was also found by Ly P et al. (2010) to have the best predictive accuracy relative to the classical equilibrium dialysis method, the one without ultracentrifugation.

Calculation of the FAI requires the measurement of total testosterone and SHBG and is defined by the following equation (Carter et al., 1983):

$$FAI = T \times 100 / S$$

Where T: is total testosterone (nmol/L) and S: is SHBG (nmol/L).

Table 1. Across group comparison of clinical features and biochemical investigations.

Clinical features (Mean ± SD)				
One-way analysis of variance (ANOVA)	**Controls**	**Hemodialysis patients**	**Renal transplanted patients**	***P* – value (two-tailed)**
Age in years	36.7 ± 7.0	38.2 ± 6.6	36.5 ± 6.3	0.61
Age of the graft in years	-	-	4.6 ± 0.4	-
Duration of Dialysis in years	-	8.2 ± 0.43	-	-
Systolic Blood Pressure*****	121.2 ± 5.1	133.2 ± 10.3	128.6 ± 11.8	< 0.0001
Diastolic Blood Pressure*****	80.6 ± 2.2	86.0 ± 5.2	84.6 ± 8.6	0.006
Biochemical investigations (Median; IQR)				
Kruskal-Wallis Test	**Controls**	**Hemodialysis patients**	**Renal transplanted patients**	***P* – value (two-tailed)**
Urea (mmol/L) *****	4.6; 1.3	22.3; 18.6	8.6; 4.7	< 0.0001
Creatinine (µmol/L) *****	79.0; 13.0	985.0; 652.0	127.0; 45.0	< 0.0001
Potassium (mmol/L)	4.2; 0.7	4.1; 1.9	4.2; 0.6	0.363
Sodium (mmol/L) ‡	140.0; 5.0	137.0; 3.0	139.5; 5.0	< 0.0001
Albumin (g/L) *****	42.0; 5.0	38.8; 5.0	39.5; 5.0	< 0.0001
Hemoglobin (g/L) *****	15.0; 1.0	11.8; 2.2	13.25; 2.4	< 0.0001
Total Testosterone (nmol/L)	15.4;6.2	17.4;10.9	15.1;9.9	0.399
SHBG (nmol/L) ******	24.2; 13.0	33.8; 20.7	31.1; 17.5	0.002
FAI% ¶	60.4; 25.5	48.4; 33.8	53.7; 34.6	0.028

SD: Standard deviation, IQR: inter-quartile range, *****: median score is significantly different among each of the three groups, ******: controls median score is significantly lower than those of hemodialysis and renal transplanted patients while there was no significant difference between the later two groups, ‡: median score of hemodialysis patients is significantly lower than those of controls and renal transplanted ones while there was no significant difference between controls and renal transplanted patients, ¶: controls median score is significantly higher than those of hemodialysis and renal transplanted patients while there was no significant difference between the later two groups , CFTV: Vermeulen equation, CFTZ: Sartorius et al. (2009) equation, and CFTNW: Nanjee-Wheeler equation.

Statistical analysis

All statistical analyses were carried out using SPSS PC+ version 13.0 statistical software and version 11.5.1.0 MedCalc software. Normality of the distribution of measured variables was assessed by Kolmogorov-Smirnov and Shapiro-Wilk tests at α level of 0.05.

Normally distributed variables were described using mean ± standard deviation (SD) and compared across the three groups using Analysis of Variance (ANOVA). Contrast testing was used to detect the pair of the normally distributed variable that had a significant mean difference. Variables that were found by normality testing to have significant departure from normality were expressed using median and inter-quartile range (IQR). Medians of the later type of variables were compared across groups using Kruskal-Wallis test followed by Pairwise analysis testing to determine the pair with significant median difference. Comparison of CFT levels derived by the three equations within each group was performed using Friedman test and Pairwise analysis. $p < 0.05$ was considered statistically significant.

RESULTS

Clinical features and biochemical investigations for the three groups were summarized in Table 1. There was no significant age difference between the three groups (p = 0.61). Expected differences in blood pressure and some routine biochemical investigations (e.g. urea, creatinine, sodium, albumin, and hemoglobin) were evident between the groups. Total testosterone did not differ significantly between the groups (p = 0.399), while SHBG level was significantly (p = 0.002) higher in hemodialysis and renal transplanted patients than in controls. FAI was significantly (p = 0.028) higher in the control group compared to the patients groups (hemodialysis and renal transplanted patients) while no significant difference was found between patients groups. Calculated free testosterone derived by CFTV and CFTZ was not significantly (p = 0.404 and p = 0.522; respectively) different across the three groups (Table 2). CFTNW gave calculated free testosterone value that was significantly different across the groups, showing that calculated free testosterone of renal transplanted men (Table 2) was significantly (p < 0.0001) higher than both control and hemodialysis groups and the later two groups had calculated free testosterone levels that were not significantly different from each other.

Detailed statistical descriptions (means, medians, SD, IQR, and percentiles) of total testosterone, SHBG, albumin, FAI, and free testosterone levels obtained by the three equations were summarized in Table 3.

The significance of the differences between calculated free testosterone values derived by the three equations within each group was illustrated in Figure 1. It was

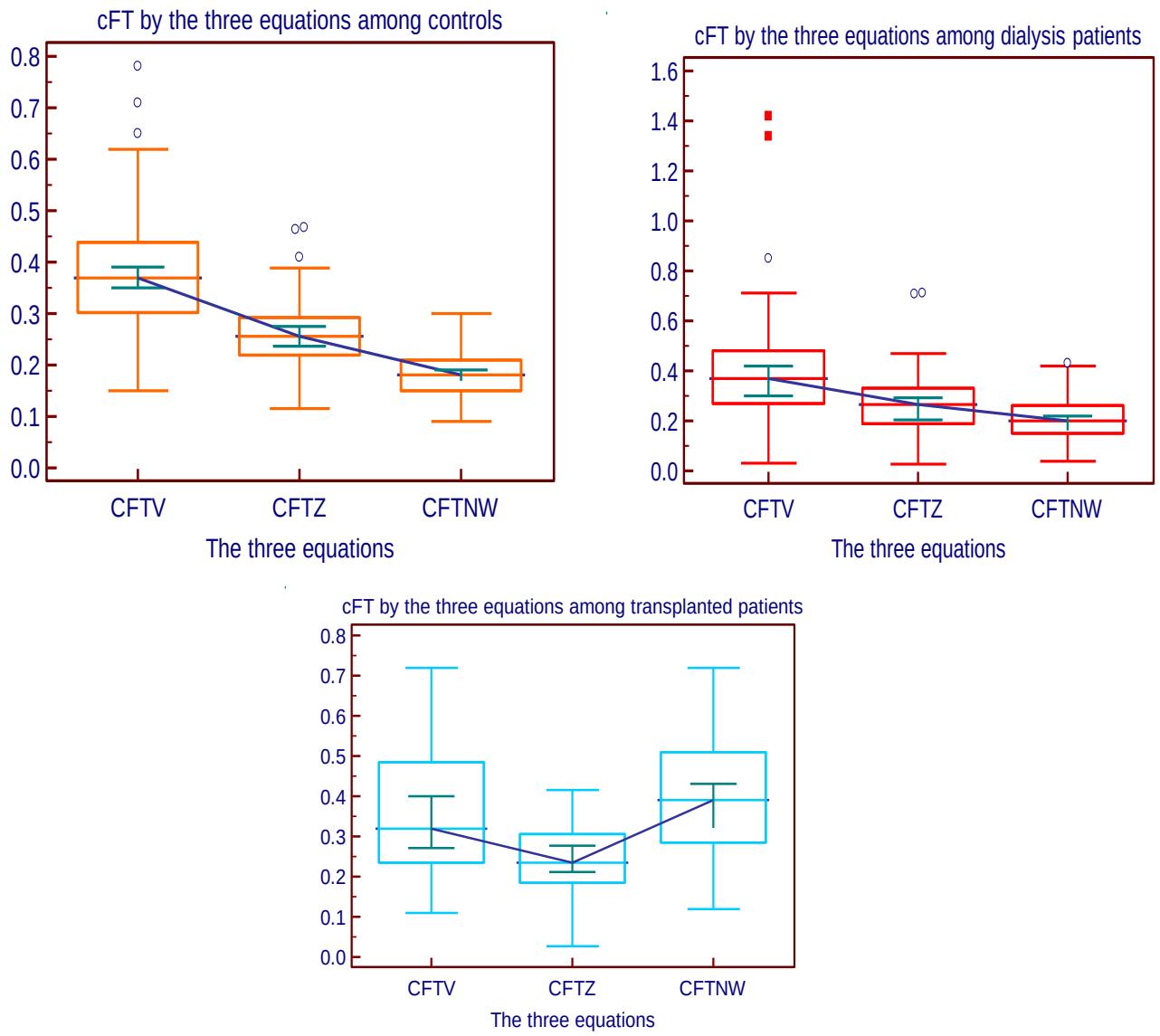


Figure 1. Free testosterone level calculated (cFT) by the three equations in each group (control, dialysis and transplant groups). Line within the box represents the median (medians are connected by blue lines), the two green horizontal lines contained with the box and surrounding the median are the upper and lower limits of 90th confidence interval of the median, lower border of the box represents 25th percentile, upper border of the box represents the 75th percentile, lower and upper whiskers represent the minimum and the maximum limits of the range, respectively. Blue-circle dots represent outlying points (that is, values larger than the upper quartile plus 1.5 times the interquartile range), and red squared dots represent extreme values (i.e. values larger than the upper quartile plus 3 times the interquartile range). CFTV: Vermeulen equation, CFTZ: Sartorius et al. (2009) equation, and CFTNW: Nanjee-Wheeler equation. P values of the differences of calculated free testosterone level obtained by the three equations were $p < 0.0001$, $p < 0.001$, and $p < 0.001$ in control, dialysis and transplant groups; respectively.

shown that in control, dialysis, and transplant groups; the significance of differences between calculated free testosterone measured by the three equations was $p < 0.0001$, $p < 0.001$, and $p < 0.001$; respectively.

Figures 2A, 2B and 2C are the illustration of those differences by Bland-Altman plot where it was shown that across the three groups CFTV was always giving calculated free testosterone values higher (mean = 0.12-0.14, 1.96 SD = 0.12-0.27) than CFTZ. It also showed that across the three groups, CFTV was always giving calculated free testosterone values higher (mean = 0.12-0.20, 1.96 SD = 0.15-0.38) than CFTNW. When it came to the difference between CFTZ and CFTNW, it showed that in control and dialysis groups, CFTZ was always giving higher calculated free testosterone values (mean = 0.07, 1.96 SD = 0.07-0.11) than CFTNW, but in transplant group this difference was reversed making CFTNW gives higher results (mean = 0.16, 1.96 SD =

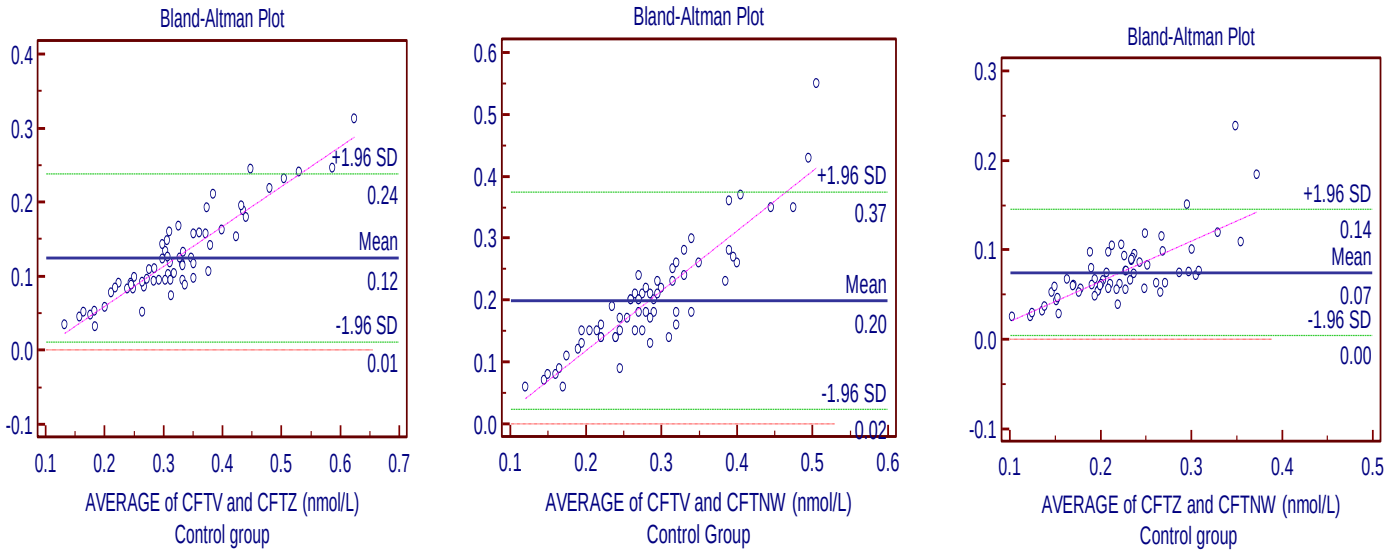


Figure 2A. Comparison of calculated free testosterone (cFT) values derived by the studied three equations among control group using Bland-Altman plot. Differences between cFT values derived from two equations are plotted against the corresponding average cFT (arithmetic mean of the two cFT values). The continuous blue line represents the mean difference, horizontal dotted green lines represent ± 1.96 SD of the differences in cFT values, the red line represents the zero(0) difference level, and the purple dotted line represents the regression line of the differences.

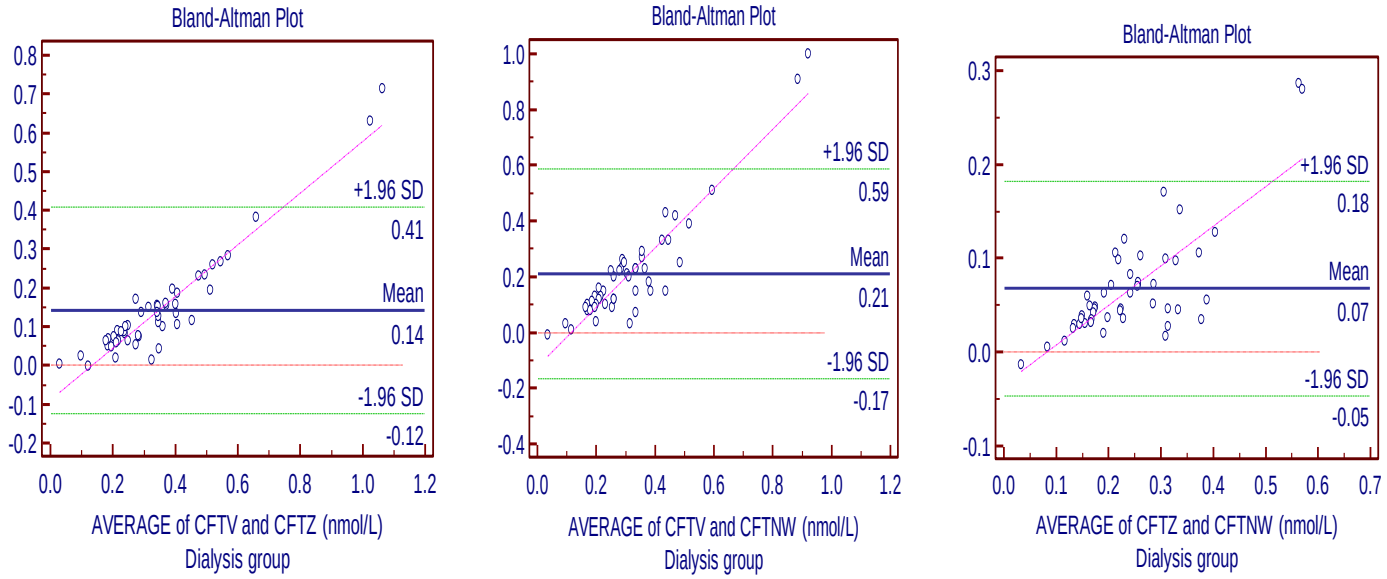


Figure 2B. Comparison of calculated free testosterone (cFT) values derived by the studied three equations among Hemodialysis group using Bland-Altman plot. Differences between cFT values derived from two equations are plotted against the corresponding average cFT (arithmetic mean of the two cFT values). The continuous blue line represents the mean difference, horizontal dotted green lines represent ± 1.96 SD of the differences in cFT values, the red line represents the zero(0) difference level, and the purple dotted line represents the regression line of the differences.

0.13) than CFTZ.

In each group, the regression equations describing the relationships between the corresponding pair of equations were summarized in Figures 2D, 2E and 2F. It shows that across the three groups the regression line between CFTV and CFTZ had a slope that varied from 1.6 to 1.9 and an intercept between - 0.03 and -0.11. In control and hemodialysis group, the relationship between CFTV and CFTNW was represented by a slope that varied from 2.24 to 2.53 and an intercept that varied

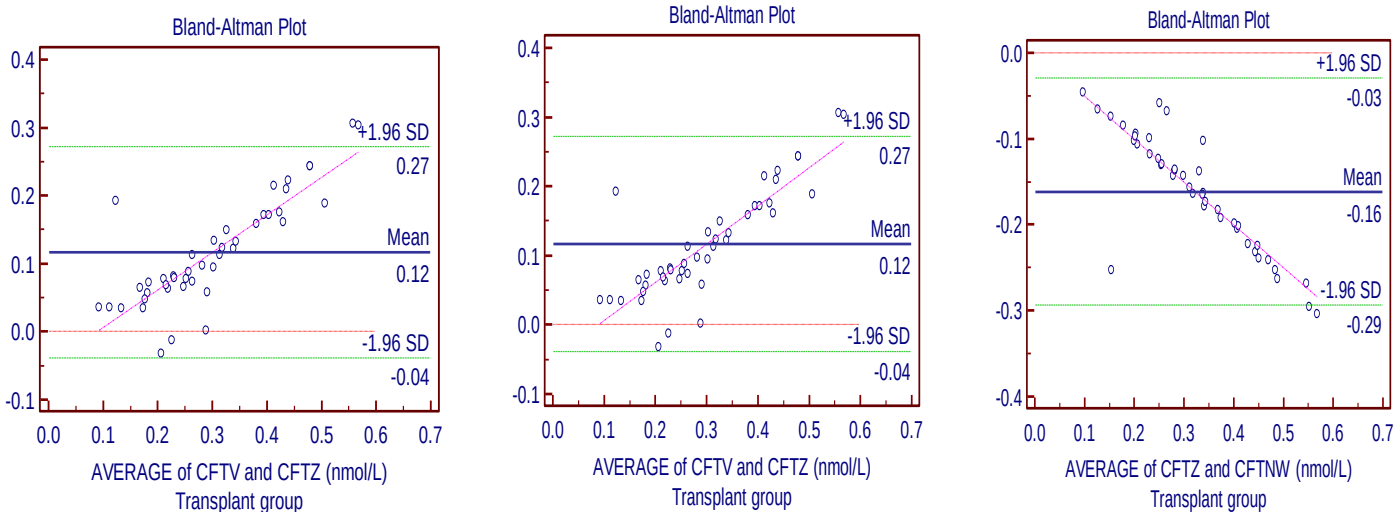
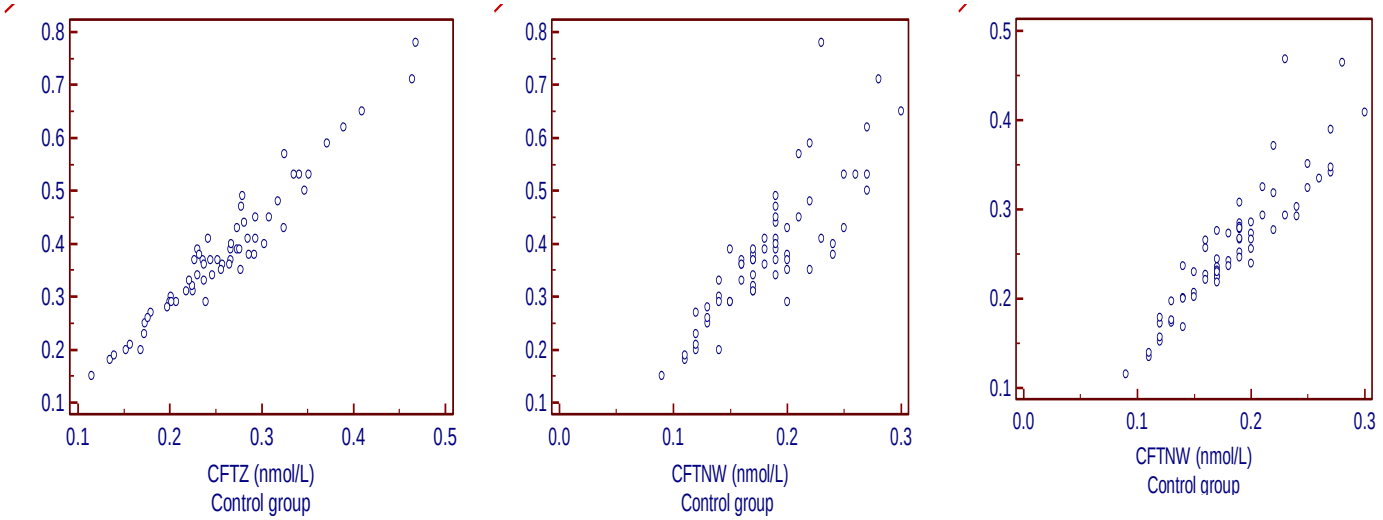


Figure 2C. Comparison of calculated free testosterone (cFT) values derived by the studied three equations among Transplant group using Bland-Altman plot. Differences between cFT values derived from two equations are plotted against the corresponding average cFT (arithmetic mean of the two cFT values). The continuous blue line represents the mean difference, horizontal dotted green lines represent ± 1.96 SD of the differences in cFT values, the red line represents the zero(0) difference level, and the purple dotted line represents the regression line of the differences.



$y = -0.05008 + 1.6734\ x,\ R^2=0.939$

Parameter	P value
Intercept	0.2347
Slope	<0.0001

$y = -0.03078 + 2.2489\ x\ ,\ R^2 = 0.7088$

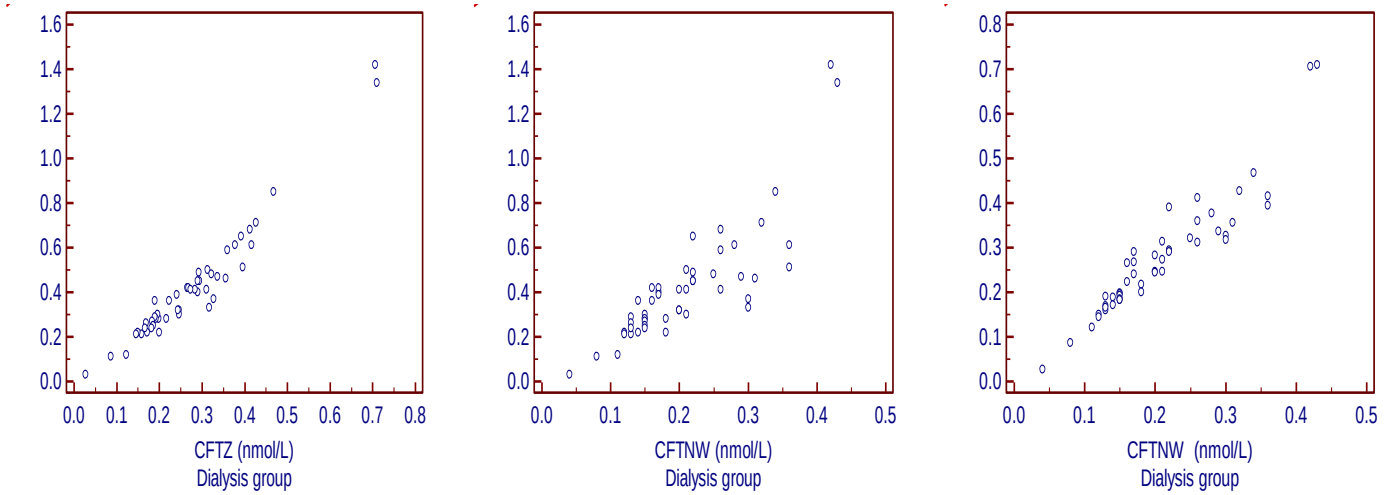
Parameter	P value
Intercept	<0.0001
Slope	<0.0001

$y = -0.00002 + 1.4066\ x\ ,\ R^2 = 0.9990$

Parameter	P value
Intercept	0.5527
Slope	<0.0001

Figure 2D. Comparison of calculated free testosterone (cFT) values derived by the studied three equations among Control group using regression analysis. R2: coefficient of determination CFTV: Vermeulen equation, CFTZ: Sartorius et al. (2009) equation, and CFTNW: Nanjee-Wheeler equation.

between - 0.03 and -0.11, while in transplant this slope dropped dramatically to 1.04 with an intercept of - 0.06. The relationship between CFTZ and CFTNW was represented in control and dialysis groups by a regression line that had a slope of approximately 1.4 and an intercept that ranged from 0 to - 0.02. In the transplant group, the slope dropped again to 0.58 with an intercept of 0.01.



$y = -0.1113 + 1.9206\ x$, $R^2 = 0.9397$

Parameter	P value
Intercept	<0.0001
Slope	<0.0001

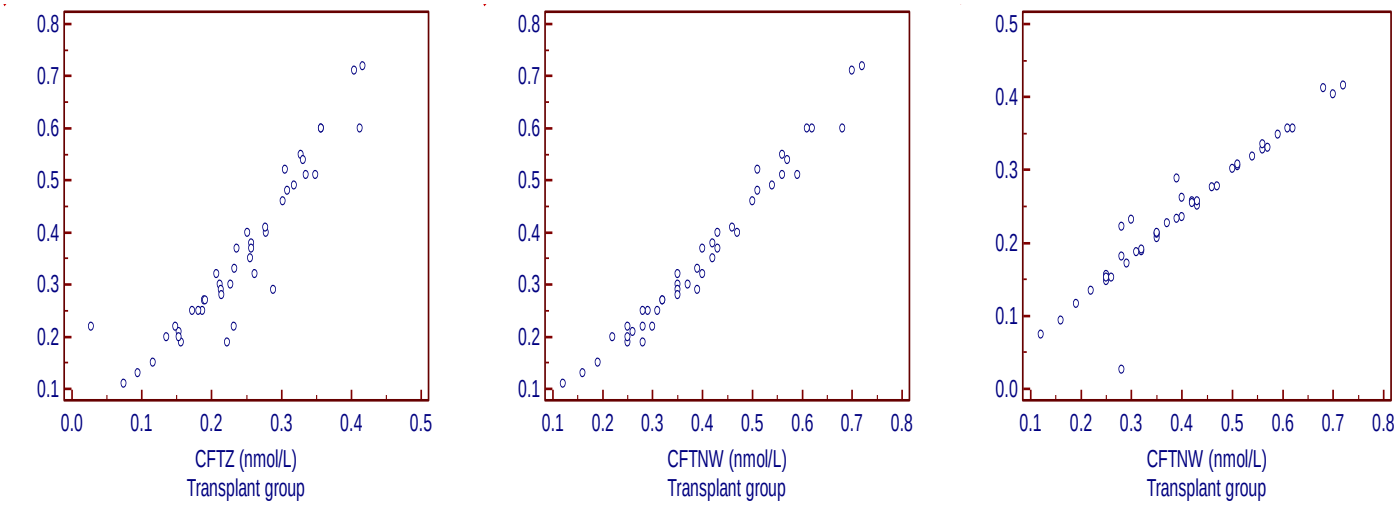
$y = -0.1077 + 2.5269\ x$, $R^2 = 0.7074$

Parameter	P value
Intercept	<0.0001
Slope	<0.0001

$y = -0.01986 + 1.4199\ x$, $R^2 = 0.8767$

Parameter	P value
Intercept	0.2618
Slope	<0.0001

Figure 2E. Comparison of calculated free testosterone (cFT) values derived by the studied three equations among Hemodialysis group using regression analysis. R2: coefficient of determination CFTV: Vermeulen equation, CFTZ: Sartorius et al. (2009) equation, and CFTNW: Nanjee-Wheeler equation.



(a)

$y = -0.03050 + 1.6077\ x$,
$R^2 = 0.8641$

Parameter	P value
Intercept	0.2347
Slope	<0.0001

(b)

$y = -0.06075 + 1.0380\ x$,
$R^2 = 0.9736$

Parameter	P value
Intercept	<0.0001
Slope	<0.0001

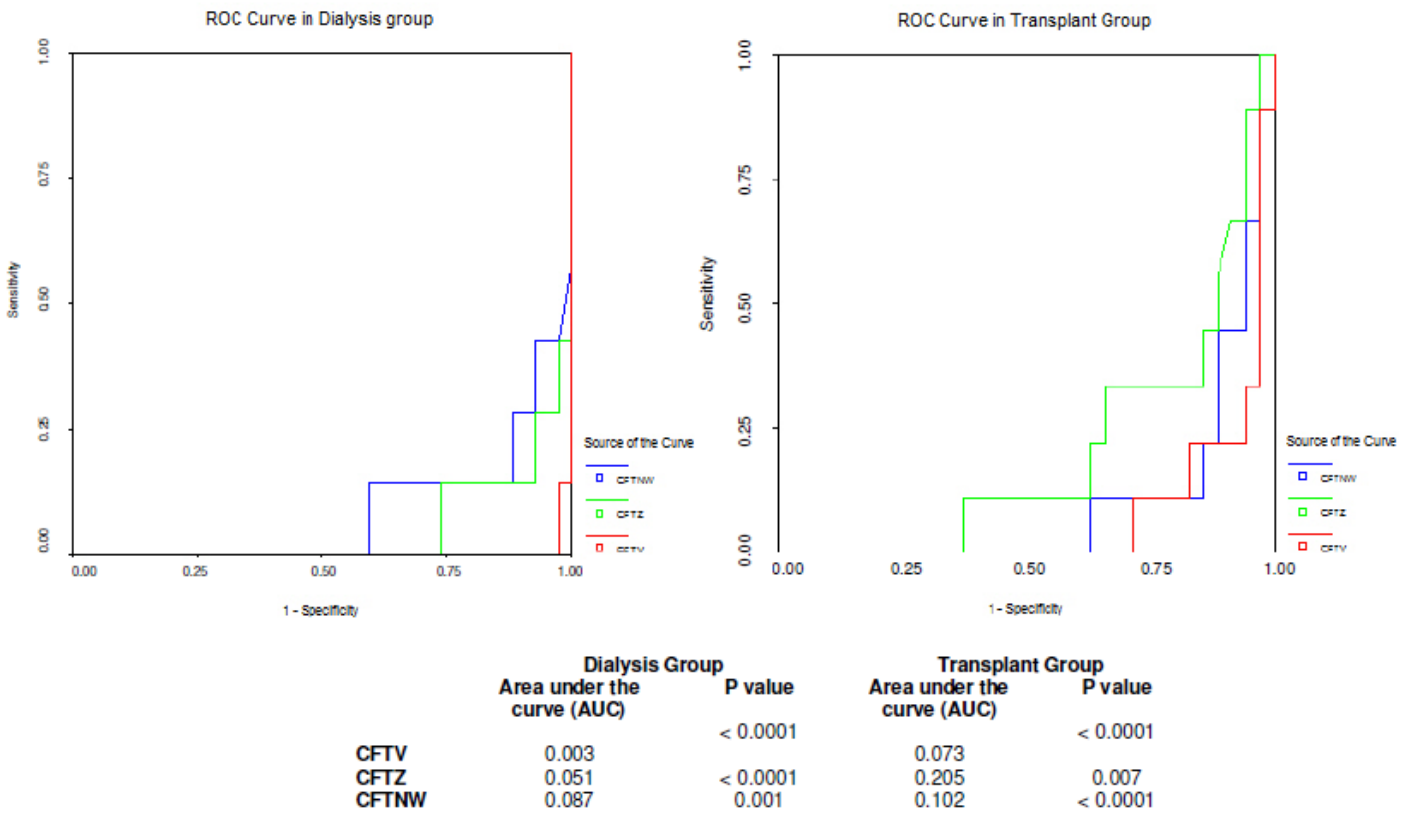
(c)

$y = 0.007081 + 0.5814\ x$,
$R^2 = 0.9135$

Parameter	P value
Intercept	0.5527
Slope	<0.0001

Figure 2F. Comparison of calculated free testosterone (cFT) values derived by the studied three equations among Transplant group using regression analysis. R2: coefficient of determination CFTV: Vermeulen equation, CFTZ: Sartorius et al. (2009) equation, and CFTNW: Nanjee-Wheeler equation.

Considering FAI as the reference method of differentiating between normal subjects and hypoandrogenic ones, receiver operating characteristic curve (ROC) for each equation in dialysis and transplant



	Dialysis Group		Transplant Group	
	Area under the curve (AUC)	P value	Area under the curve (AUC)	P value
		< 0.0001		< 0.0001
CFTV	0.003		0.073	
CFTZ	0.051	< 0.0001	0.205	0.007
CFTNW	0.087	0.001	0.102	< 0.0001

Figure 3. Receiver operating characteristic curve (ROC) of the three equations in dialysis and transplant groups with their respective area under the curve (AUC). CFTV: Vermeulen equation, CFTZ: Sartorius et al. (2009) equation, and CFTNW: Nanjee-Wheeler equation.

Table 2. Calculated free testosterone (cFT) derived by the three equations in the three groups.

	cFT (nmol/L) by the three equations (Median; IQR)			
	Controls	**Hemodialysis patients**	**Renal transplanted patients**	***P* – value (two-tailed)**
cFT(V)	0.370; 0.135	0.370; 0.215	0.321; 0.250	0.404
cFT(Z)	0.256; 0.75	0.266; 0.145	0.235; 0.125	0.522
cFT(NW) †	0.180; 0.060	0.200, 0.110	0.390; 0.225	< 0.0001

†: median score of renal transplanted patients is significantly higher than those of controls and hemodialysis ones while there was no significant difference between controls and hemodialysis patients.

groups was illustrated in Figure 3. Based on the area under curve (AUC), it was shown that the performance of the three equations was better in transplant group compared to dialysis group. CFTV ROC had the least AUC among dialysis and transplant groups. In dialysis group, the largest AUC belonged to CFNW equation, while in transplant group CFTZ had the largest AUC.

DISCUSSION

This study investigated the performance of three free-testosterone calculating equations when applied on dialysis and renal transplanted patients. Although these equations use entries (i.e. total testosterone, SHBG, and albumin) that have been standardized in terms of methods used to obtain their concentrations, they still exhibited statistically significant different ranges and medians even when applied within the same group (Tables 2 and 3). This finding reinforced the importance of establishing a distinctive reference range for each equation. It has even been recommended, due to the remarkable inter-assay variability of total testosterone and SHBG, to have different reference ranges for the same equation corresponding to the method used to assay total testosterone and SHBG. The reference range derived for Vermeulin equation (CFTV) in this study is exclusive to that equation and only valid for samples in

Table 3. Statistical description of TT, SHBG, albumin, FAI and FT calculated by the three equations.

Statistical description	CFTV (nmol/L)	CFTZ (nmol/L)	CFTNW (nmol/L)	FAI	TT (nmol/L)	SHBG (nmol/L)	Albumin (g/L)
Control group							
Percentile							
2.5	0.181	0.135	0.110	0.306	7.800	11.443	34.0
25	0.303	0.219	0.150	0.492	12.353	19.110	39.0
50 (Median)	0.370	0.256	0.180	0.604	15.400	24.200	42.0
75	0.438	0.293	0.210	0.767	18.400	32.000	44.0
97.5	0.706	0.460	0.279	1.483	25.124	45.503	48.9
Mean	0.384	0.260	0.184	0.665	15.642	25.748	41.7
SD	0.125	0.073	0.047	0.295	4.828	9.162	4.1
Dialysis group							
Percentile							
2.5	0.088	0.070	0.069	0.105	4.922	12.190	24.7
25	0.268	0.188	0.150	0.349	12.498	24.825	35.0
50 (Median)	0.370	0.266	0.200	0.484	17.420	33.810	38.8
75	0.483	0.329	0.260	0.687	23.290	45.323	40.0
97.5	1.362	0.707	0.423	2.431	39.028	77.148	45.3
Mean	0.419	0.276	0.208	0.621	18.350	36.602	37.6
SD	0.258	0.130	0.086	0.479	8.616	17.144	4.3
Transplant group							
Percentile							
2.5	0.122	0.055	0.1440	0.213	4.256	13.760	29.0
25	0.235	0.184	0.280	0.346	10.525	19.945	38.0
50 (Median)	0.320	0.235	0.390	0.537	15.090	31.145	39.5
75	0.485	0.307	0.510	0.692	20.360	37.140	43.0
97.5	0.714	0.414	0.708	1.135	29.672	92.892	56.8
Mean	0.358	0.241	0.403	0.558	15.787	32.409	40.0
SD	0.156	0.090	0.148	0.255	6.321	18.828	4.0

TT: total testosterone, FAI: free androgen index, SHBG: sex-hormone binding globulin, SD: standard deviation, and calculated free testosterone level calculated by CFTV: Vermeulen equation, CFTZ: Sartorius et al. (2009) equation, and CFTNW: Nanjee-Wheeler equation.

which total testosterone and SHBG has been measured using Architect i2000 CMIA method. If another lab is relying on Vermeulin equation to obtain calculated free testosterone level and uses Architect i2000 CMIA method to assay SHBG, but assays total testosterone on another immunoassay format like Elecsys 2010, for instance, this lab can not rely on our derived CFTV reference range. Here is an example; if a sample of 25 years old man reads total testosterone of 12.8 nmol/L and SHBG of 45.5 nmol/L on Architect i2000, calculated free testosterone level will be 0.216 nmol/L which is normal. If the same sample is assayed on Elecsys 2010, it would approximately read 7.0 nmol/L. If the Elecsys 2010 total testosterone result is entered in CFTV, you will have calculated free testosterone level of 0.111 nmol/L which, when compared to our CFTV normal reference range, would be falsely considered as hypoandrogenic. This example illustrated one of the limitations of using equations to calculate free testosterone.

Within group comparison of calculated free testosterone values obtained by each equation (Figure 1) showed that these equations gave calculated free testosterone levels that were significantly different from each other within all three groups. That comparison also showed that in control and dialysis groups, CFTV gave higher result (mean difference = 0.12 nmol/L in control and 0.14 nmol/L in dialysis group) than that of CFTZ that in turn gave a higher calculated free testosterone level

(mean difference = 0.07 nmol/L in both control and dialysis group) than CFTNW (Figures 2A, 2B and 2C). In other words, in control and dialysis group this order was maintained: CFTV > CFTZ > CFTNW. In renal transplant group, the relation of CFTV > CFTZ did not change, but unexpectedly CFTNW equation gave the highest calculated free testosterone values among the three equations changing the order to be CFTNW > CFTV > CFTZ. This dramatic change was also evident in Figures 2D, 2E and 2F, where the slope of the regression curve describing the relationship between CFTV and CFTNW dropped from 2.25 in control and 2.53 in dialysis to 1.04 in transplant group. This slope change was even clearer when we looked at the three slopes of the regression lines describing the relationship between CFTZ and CFTNW, where the slope has changed from 1.41 in control and 1.42 in dialysis (almost identical slopes) to 0.58 in transplant group. Both CFTZ and CFTNW rely only on total testosterone and SHBG to calculate free testosterone level (no entry for albumin level is needed) and they are both empirically derived, but CFTZ has been validated by ultra-filtration and equilibrium dialysis reference methods, while CFTNW was validated by gel filtration method. However, CFTZ had consistent performance in terms of reporting calculated free testosterone values that are consistently lower than CFTV in all three groups, while CFTNW gave lower calculated free testosterone values than both other two equations in control and dialysis groups, but started to give higher results than both of them when applied to transplanted patients. This discrepancy manifested itself in another way when we considered FAI as a reference method for diagnosing hypoandrognic subjects and compared the diagnostic utility of the three equations with it (Figure 3). The diagnostic utility of each equation was represented by ROC curve where the AUC was proportional to the respective equation. It was clear that the three equations had better performance when applied to transplanted patients compared to dialysis ones. It was also clear that in both dialysis and transplant groups, CFTV had the worst performance among the three equations.

The best performance in dialysis group was that of CFTZ, while in transplant group CFTNW had the best performance. When ordering these three equations based on the corresponding AUC we get in dialysis group this order: CFTNW > CFTZ > CFTV, while in transplant group the order is: CFTZ > CFTNW > CFTV. This unexplained change of order is another form of discordance we found between these equations.

When CFTV, CFTZ, CFTNW and FAI were used to compare the three groups (control, dialysis, and transplant groups) according to their corresponding calculated free testosterone levels, CFTV and CFTZ did not show any significant difference between these groups (Table 2). On the other hand, CFTNW ordered them as transplant group > (control group = dialysis group) and FAI ordered them as: control group > dialysis group > transplant group. It is obvious that even after standardizing the methods used to derive their required entries, these equations gave discordant results when compared to each other and when compared to FAI. Since we did not measure the exact free testosterone level using a reference method, we could not tell for sure whether FAI or one of the CFT equations was giving the correct order.

In conclusion, calculation of free testosterone rather than its laborious direct measurement is a practice that has recently been gaining more popularity especially after been advocated by some researchers and after the emergence of more calculating equations. Some of these equations are supported by studies that validated them against reference methods. However, most of these studies if not all have experimented the validity of these equations on normal subjects. This study showed that when applying some of these equations on diseased subjects such as dialysis and renal transplanted patients, a significant discrepancy between them is found, which demands that such calculation practice should not be carried out in diseased people without prior validation of the equation to be used. Without comparison against a reference method, we could not prove the superiority of an equation over another or whether FAI was superior to those equations, however, the absence of such comparison does not invalidate the discrepancy found in this study.

We hope that this study would encourage other researchers to explore the validity of other equations in other diseased subjects and would alert healthcare givers about the use of these equations on patients without validating them first.

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Abbreviations: SHBG, Sex-hormone binding globulin; **LH,** luteinizing hormone; **FSH,** follicle stimulating hormone; **FAI,** free androgen index; **HPGA,** hypothalamic-pituitary-gonadal axis; **CV,** coefficients of variation; **CFT,** calculated free testosterone; **SD,** standard deviation; **ANOVA,** analysis of variance; **IQR,** inter-quartile range; **ROC,** receiver operating characteristic curve; **AUC,** area under the curve; **CMIA,** chemiluminescent microparticle immunoassay method.

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Full Length Research Paper

Enterovirus outbreak among preterm infants in Singapore General Hospital: Level II nursery

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To describe the demographic characteristics, clinical presentations and outcome of neonatal enterovirus infection and to evaluate infection control measures effective in preventing the spread of infection. Detailed perinatal history, demography, neonatal conditions and laboratory investigations were retrieved retrospectively from 5 Enterovirus positive patients in the level II nursery of the Singapore General Hospital in October 2010. Five premature neonates tested positive for Enterovirus during the outbreak. All infected neonates presented with lethargy, 4 (80%) poor suck, 4 (80%) apnea, 2 (40%) poor perfusion and 1 (20%) had pyrexia. Enterovirus was positive on PCR analysis of the stool specimen in four infected neonates and on spinal fluid in one neonate. All infected neonates required respiratory support; 3 needed continuous positive airway pressure (CPAP), one required SIPPV and another needed HFOV. Inotropes was needed in 1(20%) infant and severe thrombocytopenia was documented in 3 infected neonates. The neonate who required High frequency oscillatory ventilation (HFOV) and inotropic support died on day 5 of illness. Four (80%) neonates recovered 5 days following the onset of illness. Neonatal enterovirus infection can lead to morbidity and even death. Pertinent history of exposure, early recognition, timely intervention and appropriate infection control measures are necessary to prevent dissemination of infection.

Key words: Enterovirus, coxsackie type B5, neonatal enterovirus, ev in Singapore.

INTRODUCTION

Neonates have immature immune system and are at higher risk for serious complications of bacterial and viral infections, including enteroviral diseases. Evaluating neonates with mild and nonspecific symptoms that are consistent with upper respiratory or viral processes can be challenging. Poliovirus, the prototypical Enterovirus can cause a subclinical infection or illness such as aseptic meningitis or paralytic poliomyelitis. The non-polio viruses which commonly infect infants include group A and B Coxsackieviruses, Echoviruses and Enteroviruses. Infected neonates can have symptoms ranging from self-limited disease to generalized multi-system organ failure and sepsis (Joki-Korpela et al., 2001; Kaplan et al., 1983; Krajden and Middleton, 1983). Enteroviral disease in neonatal period may be acquired antenatally, intrapartum or postnatally. Other common mode of transmission in infancy includes fecal-oral route and oral-oral contamination; swimming pools, wading pools, and contaminated hands (Modlin, 1986, 2000). Enterovirus survives on surfaces for long periods of time, allowing for transmission by fomites such as toys, books and doorknobs.

Typically, the onset of a generalized enteroviral infection occurs at 3 to 5 days post contact, though some may present with a diphasic illness characterized by 1 to 7 days of recovery between initial presentation and disease progression. Early symptoms of an enteroviral infection may include lethargy, decreased feeding and transient respiratory complaints. Substantial mortality rates have been reported, and long-term sequelae may occur among survivors. Risk factors and clinical features associated with severe disease include absence of neutralizing antibody to the infecting serotype, maternal illness prior to or at delivery, prematurity, illness onset within the first few days of life, multi-organ disease,

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severe hepatitis, positive serum viral cultures, and specific infecting serotype (e.g. group B Coxsackie viruses and Echovirus) (Lin et al., 2003). Viral culture and PCR are effective methods for the detection of Enterovirus. Advantages of using a PCR over a viral culture included faster results and improved sensitivity; however, the use of PCR is laboratory dependant (Ahmed et al., 1997). At this time, treatment of enteroviral disease in neonates is supportive, including addressing complications of diseases such as hepatitis and myocarditis, and initiating antibiotics such as ampicillin, gentamicin or cefotaxime along with the consideration of vancomycin in the very ill-appearing infant. Use of IVIG and Pleconaril in infected neonates as therapy remains experimental (Abzug et al., 1993, 1995, 2004).

Outbreak of enterovirus infection occurs sporadically in neonatal units. We report an enteroviral outbreak at a level II nursery in October 2010. The aim of this case report is to study the clinical presentation and morbid consequences of enterovirus infection as well as to highlight the importance of detailed history taking in early detection of the disease and the role of strict infection control measures in limiting the spread of infection.

MATERIALS AND METHODS

Case records of the 5 infected infants were retrieved. The 2 infected infants (index cases) were in the level II nursery since birth due to prematurity and the rest of the premature neonates were in level II nursery awaiting weight gain and good suck. The demographic characteristics, clinical presentation, laboratory and radiographic tests as well as clinical progress of these infants were collated and compiled. Infection control measures over the period of the outbreak were reviewed and evaluated.

Description of outbreak

The outbreak occurred in the level II nursery in the Neonatal Department at the Singapore General Hospital, Singapore on October 21, 2010. The Level II nursery is a step down unit which can accommodate a total of 18 neonates. The nursery comprises of 2 rooms (Appendixes 1 and 2), room A which can accommodate 10 patients and room B comprised of a foyer and 4 cubicles which can accommodate another 8 patients. A total of 6 inpatients were admitted to room A and another 6 infants were admitted to room B on the day of presentation of the first index patient.

The first index patient was a day 6 infant, 1st of twin admitted to room A. Within 12 h of presentation, the 2nd twin who was nursed beside her was also symptomatic with feeds intolerance and poor perfusion. These two index patients were approximately 15 m away from the other infected patients who were nursed in room B at the time of presentation of the index patients. The two infected patients nursed in room B presented with frequent episodes of desaturation and lethargy two days following the onset of illness in the index patients. The 3rd infected patient was a patient who was discharged from room B 5 days after the onset of enterovirus infection in the index patients. This infant developed fever 2 days after discharge and was re-admitted to another hospital for pyrexia. All infected infants were stable in room air prior to onset of signs and symptoms.

Detailed history of maternal well-being re-obtained from parents of the index patients 2 days following the onset of illness reviewed that an older 2½ y/o sibling was diagnosed to have Hand Foot Mouth Disease a week prior to mother's delivery. Mother's stool tested on day 8 of twin's illness was negative for EV PCR, EV 71 RNA and EV Panel.

Index case

The index cases, a set of twins located in room A, were 6 day old at presentation. These twins were delivered vaginally at 34/52 gestation with the birth weight of 2005 g (Twin 1) and 2110 g (Twin 2) respectively. They were admitted for prematurity and were stable till day 6 of life when they developed poor suck, lethargy, poor perfusion and apnea requiring NICU care. Septic screen conducted included aerobic blood and urine culture and chest radiography. Stool specimen analyzed on day 2 of illness was positive for Enterovirus on PCR. Stool culture confirmed the presence of Coxsackie virus type B5 in both twins. Coagulation profile was prolonged in both infants resulting in need for multiple blood product transfusions. Twins developed respiratory distress requiring nasal CPAP (NCPAP). Illness stabilized with supportive care and twins were eventually discharged well on day 26 of life.

Spread of enterovirus infection

Two days following the onset of symptoms in the twins, two other patients in room B manifested with respiratory depression and were apneic and lethargic. Both infants were symptomatic within hours apart and both were transferred to the NICU for respiratory support and to evaluate for signs of sepsis. The 1st infected infant who was born at 32 weeks gestation with the birth weight of 1365 grams was 17 days old when he presented with recurrent apnea and lethargy. This infant needed Synchronized Intermittent Positive Pressure Ventilation (SIPPV) deteriorating to need High Frequency Oscillatory Ventilation (HFOV). This infant was also hypotensive needing inotropic support and had severe coagulopathy requiring multiple blood product transfusions. Stool analyzed was positive for enterovirus on PCR. Blood culture was positive for *Klebsiella* and ETT culture was positive for *Klebsiella* and *Sternophila trophomonas*. In spite of maximal support, infant continued to deteriorate and eventually died on day 5 of illness.

The 2nd infected infant was born at 31 weeks gestation with the birth weight of 1490 g. She was 21 days old when she presented with apnea and lethargy. Infant was ventilated and supported for respiratory depression and coagulopathy with positive response. Stool PCR was positive for Enterovirus and stool culture was positive for Coxsackie virus type B5. Blood culture and culture of the endotracheal aspirate were negative for bacterial growth. She progressed positively with support and was transferred out of NICU 5 days post onset of illness.

The third infected infant presented with fever 2 days following discharge from room B. This infant, an ex-preterm child born at 25 weeks gestation was discharged 5 days following the onset of illness in the index patient at the post menstrual age of 45.4 weeks. Septic screen done confirmed the presence of Enterovirus on PCR conducted on his spinal fluid confirming the diagnosis of Enterovirus meningitis. Infant remained stable with symptomatic care comprising of CPAP and antibiotic. He was discharged well 5 days later.

Infection control measures

Both index patients and 2 infected infants were cohorted in the NICU. New admissions to NICU were restricted to a separate wing

Table 1. Patient demography.

Patient/Place admitted	Gestational age (weeks)	Birth weight (gm)	Age of onset (days)	Previous lung condition	Respiratory support prior to illness	Reasons for stay in Level II nursery
Index 1(Twin 1) Room A	34	2005	6	normal	Room air	Premature newborn
Index 2 (Twin 2) Room A	34	2110	6	normal	Room air	Premature newborn
Infected Patient 1 / Room B	32	1365	17	HMD	Room Air	Growing premature neonate Awaiting good suck
Infected Patient 2 / Room B	31	1490	21	Pneumonia	Room Air	Growing premature neonate Awaiting good suck
Infected Patient 3 / Room B	25	830	145	HMD and CLD	Room Air	Growing premature neonate Awaiting good suck

of the NICU. During this period, the nurse to patient ratio in NICU was maintained at 1:2 and 1:4 in the NICU and level II nursery respectively. Exposed infants nursed in the level II nursery were cohorted and discharged home directly from the nursery when medically fit with strict advice to observe for signs and symptoms of infection. No new admissions were allowed to the level II nursery for 3 weeks following the diagnosis of the last infected patients. During the outbreak, standard infection control policies and hand hygiene were reinforced. Strict hand hygiene practice using soap and water or alcohol hand-rub before and after handling of each patient and their surroundings was implemented and audited judiciously. The staff assigned to nursing the Enterovirus positive cases was not assigned to nurse the non-infected asymptomatic infants.

RESULTS

The median birth weight of the 5 infected infants was 1560 g (range from 830 to 2110 g), with median gestational age of 31 weeks (range from 25 to 34 weeks) and median postnatal age of 39 days (range of 6 to 147 days) at presentation, (Table 1).

All infected neonates were lethargic at presentation with 80% having apnea, 40% with poor suck, 40% having poor perfusion and 20% with pyrexia. All infants required respiratory support; 3 of whom needed CPAP, 1 required SIPPV and 1 needed HFOV. Inotropic support was required in 1 infant. Severe thrombocytopenia and prolonged coagulation profile were documented in 60% of infected neonates, all of whom required multiple blood product transfusion, (Table 2).

Stools were positive for Enterovirus on PCR in 4 (80%) infected neonates while one (20%) infant was positive for Enterovirus on spinal tap fluid. Three infants were positive for Coxsackie virus type B5 on the stool culture. Nasopharyngeal aspirate culture for respiratory viruses was assayed from all four infants who presented with respiratory symptoms, however cultures was negative for the panel of common respiratory viruses. ETT culture performed on the ventilated infants confirmed the presence of *Klebsiella* and *Sternotrophomonas maltophila* in 1 infant. Aerobic blood culture was assayed on all five infants, of whom 1 infant was positive for *Klebsiella*. This same infant was also positive for *Klebsiella* in his endotracheal aspirate confirming the presence of superimposed *Klebsiella septicemia*. This infant required inotropic support and high frequency oscillatory ventilation. Antibiotic therapy instituted yielded no positive response and infant subsequently died on day 5 of illness. Antibiotics were also instituted to the other 4 symptomatic infants whilst awaiting the aerobic culture results. Illness resolved in these infants 5 days following the onset of illness, resulting in a mortality rate of 20%, (Table 2).

DISCUSSION

This report described the pattern of transmission of enterovirus infection among neonates nursed in a level II nursery. Spread and spectrum of illness among infected neonates emphasized the potential for enterovirus infection to cause widespread illness with substantial morbidity among this highly susceptible population.

Enterovirus can be classified into polio and non-polio enterovirus infection. The latter is common in the neonatal period, manifesting with non specific signs and symptoms. Unlike older children and adults, some neonates with enterovirus infection can progress to multi-system disease and death. Multiple clinical syndromes varying from asymptomatic viral shedding, nonspecific febrile illness, aseptic meningitis, hepatic necrosis, coagulopathy and myocarditis are seen with neonatal enteroviral infection. Similar to reports by Huang et al. (2010), our patients presented with varying severity of the illness.

The mortality rate of 20% in our outbreak is similar to that reported by Kaplan et al. (1983); Krajden and Middleton

Table 2. Clinical presentation and progress of affected neonates.

Patient /Place admitted	Clinical features	Investigations	Respiratory support during illness	Other treatment	Duration of symptoms (days)
Index 1 /Room A	Lethargy, Poor suck, Poor perfusion, Apnea	Stool EV PCR - positive Stool c/s - positive for Coxsackie virus type B5 NPA - negative for respiratory virus Blood c/s - no growth Coagulation profile - prolonged Thrombocytopenia	Nasal CPAP	Antibiotics Multiple blood product transfusion	5
Index 2 /Room A	Lethargy, Poor suck, Poor perfusion, Apnea	Stool EV PCR - positive Stool c/s – positive for Coxsackie virus type B5 NPA - negative for respiratory virus Blood c/s - no growth Coagulation profile – prolonged Thrombocytopenia	Nasal CPAP	Antibiotic Multiple blood product transfusion	5
Infected Patient 1 /Room B	Lethargy, Apnea	Stool EV PCR – positive NPA - negative for respiratory virus ETT c/s – positive for Klebsiella and Sterno trophomonas Blood c/s – positive for Klebsiella Coagulation profile prolonged	SIPPV+VG→ HFOV	Antibiotic, Multiple blood product transfusion	Died on day 5 of illness
Infected Patient 2 /Room B	Lethargy, Apnea	Stool EV PCR - positive Stool c/s - positive for Coxsackie virus Type B5 NPA - negative for respiratory ETT c/s - no bacterial growth Thrombocytopenia	SIPPV+VG	Antibiotic, Multiple blood product transfusion	5
Infected Patient 3 /Room B	Lethargy, Pyrexia	Stool EV PCR - negative EV 71 - not detected Blood c/s - negative for bacterial growth CSF Culture - positive for EV	NCPAP	Antibiotic	5

1983). Although the majority of infections in the neonate are benign, high index of suspicion will expedite efficient management. Physicians should also recognize the clinical manifestations and risk factors for severe disease to anticipate complications and to implement intensive management of infants at high risk of adverse outcomes. No sex predilection for enteroviral infection has been reported, however 3 of our infected patients (60%) were

females.

Enterovirus infection can result in significant morbidity in premature infants. In this outbreak, two out of 5 infants (40%) developed severe respiratory distress requiring mechanical ventilation. As seen in our patients, the spectrum of respiratory compromise can range from mild to severe. Enteroviral infections may lengthen the stay of hospitalization of infected infants. Indeed, 1 of our infant was re-admitted following exposure to the enteroviral infection outbreak. The mean duration of symptoms among our infected infants was 5 days. This is comparable with reports by Huang et al. (2010) and Nino et al. (2006).

In our nursery, the immediate identification of symptoms with a high index of suspicion and implementation of strict infection control measures controlled the spread of infection despite the fact that many infants were exposed. Strict cohorting practice implemented early and lasting for a period of 3 weeks contributed to the limitation of the disease in the nursery. However, cohorting of exposed patients may not be possible in units with limited space and overcrowding will promote transmission of an outbreak. Given that the index patients were symptomatic within the week of delivery, it is postulated that the infection was vertically transmitted from their mother who cared for an older sibling who was diagnosed clinically to have hand-foot-mouth disease a week prior to her delivery.

The 3 other infants were infected horizontally within the nursery. Enteroviruses are transmitted predominantly via the fecal-oral route, however, Coxsackie virus A21, which is spread mainly by respiratory secretions and Enterovirus 70 are known to spread via respiratory secretions and shed in tears and spread via fingers and fomites respectively. Three of our infected patients were positive for Coxsackie virus type B5. These infants possibly acquired the virus through respiratory secretions and/or tears which was spread via fingers of health care staff.

It is known that Enteroviruses upon entry into the oropharynx would replicate in submucosal tissues of the distal pharynx and alimentary tract. Viral particles are subsequently shed in the feces and in upper respiratory tract secretions for days prior to symptom onset. The average incubation period is 3-10 days, during which time the virus migrates to regional lymphoid tissue and replicates further. Minor viremia results and dissemination to target organs follows. Viral replication in target organs produces the major viremia with possible secondary seeding of the CNS. Potential target organs include the skin and the central nervous system. Infectious virus is shed from the upper respiratory tract for 1-3 weeks and from the feces for 3-8 weeks.

Among the infected patients, the potential risk factor for enterovirus infection noted was prematurity. All infected infants were born prematurely but were apparently well till onset of signs and symptoms of infection. Considering how fast the deterioration of patients with enterovirus infection was, it is indeed important to have a high index of suspicion so that proper management strategy and control measures can be implemented.

Conclusion

Enterovirus infection is a life threatening viral infection if not detected early. It can present with non specific signs and symptoms in infants, hence high index of suspicion and early diagnosis is necessary to prevent an outbreak. Proper and complete history taking is necessary to be able to elicit information that can be overlooked by a non medical practitioner like parents. This outbreak reinforced the benefits of strict contact precaution and hand hygiene technique among all involved in care of vulnerable patients. It also enlightened the understanding of physicians that enteroviral infections should be taken seriously and that rapid deterioration leading to death can happen in neonates.

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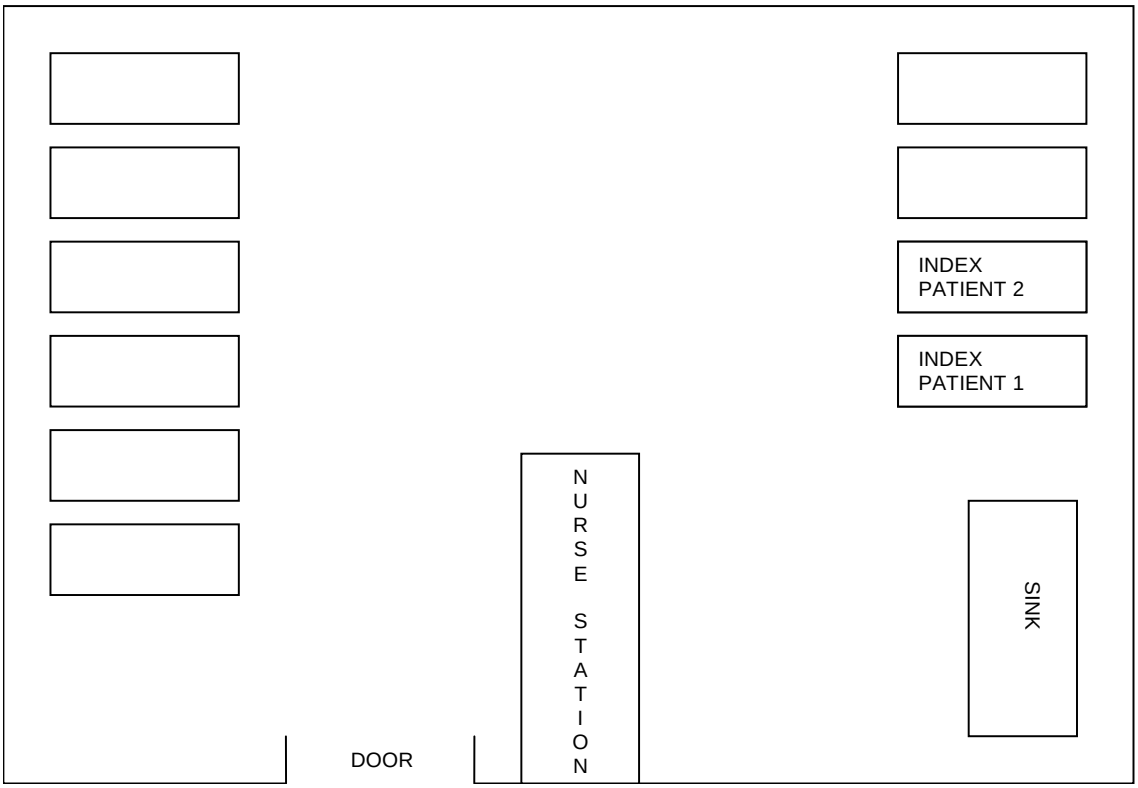
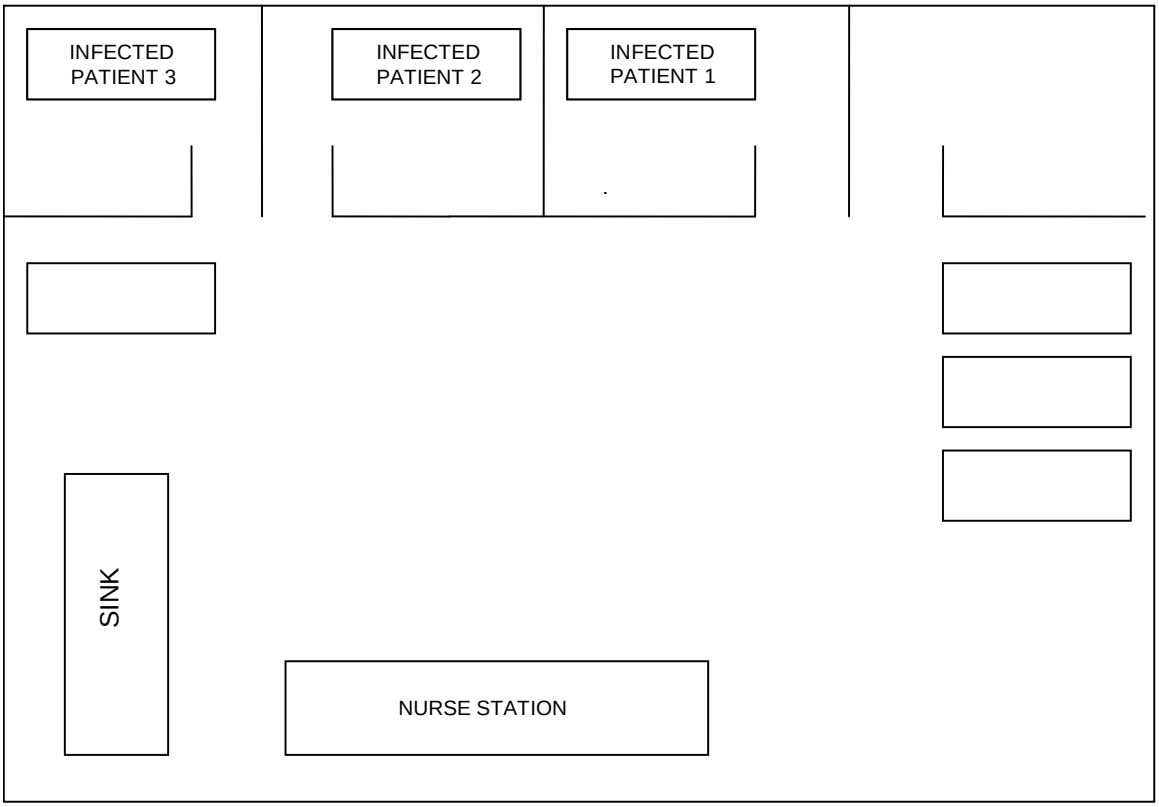
Appendix 1. Room A.

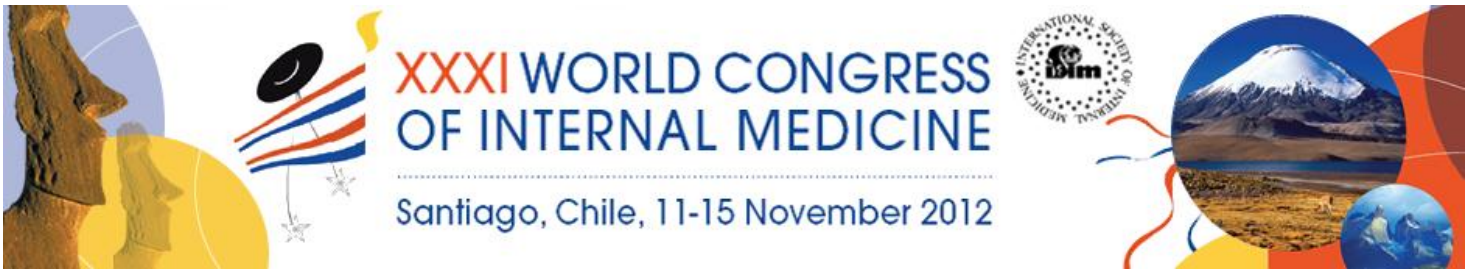


Appendix 2. Room B.



UPCOMING CONFERENCES

XXXI World Congress of Internal Medicine
Santiago, Chile, 11-15 November, 2012



European Society of Intensive Care Medicine - LIVES 2012 Lisbon, Portugal, 13-17 October, 2012

SoCRA 21st Annual Conference
Las Vegas, Nevada - September 21- 23, 2012

Conferences and Advert

August 2012
4th EuCheMS Chemistry Congress (ECC), Prague, Czech Republic, 26 Aug 2012

September 2012
8th Congress of Toxicology in Developing Countries (CTDC8), Bangkok, Thailand, 10 Sep 2012

2013

March 2013
11th International Conference of Chemistry & its Role in Development, ElSheikh, Egypt, 11 Mar 2013