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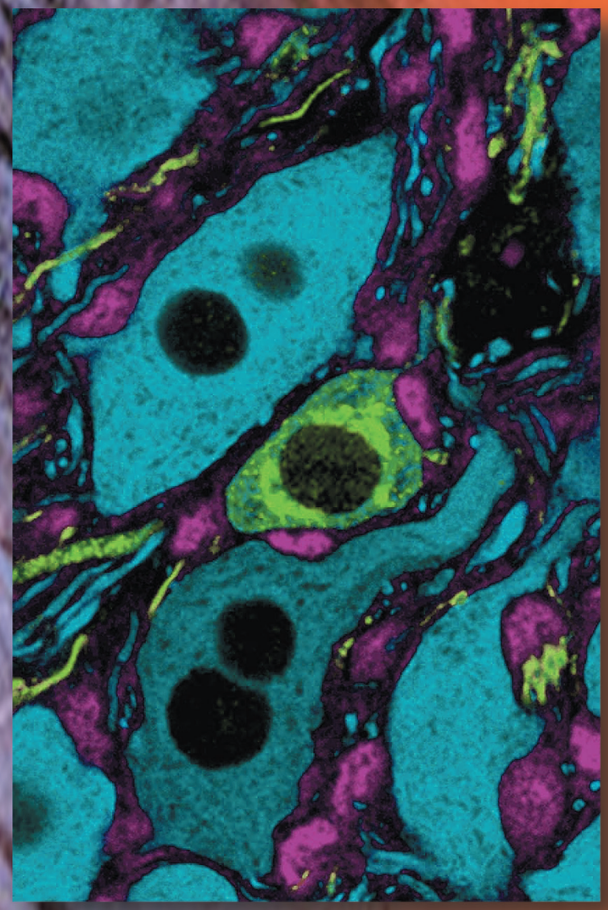

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CONTENTS

February & May 2019 Vol. 40 No. 1 & No. 2

Original Articles

Haematology Update

Regular Features

Advertising Index

Instructions to authors *are available on the AIMS website www.aims.org.au*

ORIGINAL ARTICLE

Thyroid disease in pregnancy

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Abstract

The universal screening of thyroid function in all pregnant women is contentious. However, when testing is performed, the correct classification of maternal thyroid status is essential to prevent adverse maternal and foetal outcomes, both in hypo- and hyper-thyroid illnesses. The preferred thyroid function screening test is measurement of thyroid stimulating hormone (TSH) levels. Due to the physiological changes of pregnancy, particularly the increase in concentration of thyroid binding globulin (TBG), and the weak stimulating effect of human chorionic gonadotropin (hCG) on the TSH receptor, the reference ranges of TSH determined in the general population may not be applicable in pregnancy. This review will examine the evidence for the use of trimester specific TSH ranges in pregnancy and how these ranges were obtained. This will include reviewing the current guidelines for TSH testing in pregnancy issued by the Endocrine Society of America, American Thyroid Association, and the European Thyroid Association as well as Obstetric Guidelines of The Royal Australian and New Zealand College of Obstetricians and Gynaecologists. This review will inform the need and provide the framework for conducting a trimester specific TSH reference range determination.

Keywords: universal screening, maternal thyroid status, thyroid binding globulin, human chorionic gonadotrophin

Introduction

The thyroid gland sits in the anterior neck and normally comprises two small lobes connected by an isthmus at the midline. The thyroid parenchyma is comprised of follicles which produce and store thyroglobulin, the precursor to the thyroid hormones thyroxine (T4) and triiodothyronine (T3), using a process that requires iodides produced from iodine (Kaplan and Pesce 2010). The physiological effect of thyroid hormones is to control cellular metabolism, having actions on metabolism, cardiovascular and nervous system (Kaplan and Pesce 2010).

Thyroid hormone secretion is controlled via the hypothalamic-pituitary-thyroid axis. The circulating levels of T4 and T3 feedback to the anterior pituitary and hypothalamus to regulate the secretion of TSH from the anterior pituitary which is itself under the control of hypothalamic thyrotropin-releasing hormone (Figure 1). Thyroid hormone synthesis by the follicular cells is outlined in Figure 2.

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The daily secretion of thyroid hormone includes 80-100µ of T4 and around 7µ of T3. All T4 production occurs within the thyroid gland. Up to 80% of T3 is produced by extrathyroidal deiodination of T4 predominately by the liver and kidney, however many other tissues possess the capacity to produce some T3 (Kaplan and Pesce 2010).

On release into the circulation, T4 and T3 are primarily bound to one of three binding proteins as outlined in Table 1. It is the unbound free fractions that are readily available for cellular uptake, with T3 being the most physiologically active. The physiologically-available free fractions are measured in the laboratory when assessing thyroid function.

The measurement of TSH is the current screening test for thyroid disease. Table 2 summarises the features and prevalence of thyroid disease in Australia.

Primary hyperthyroidism is a disease state where thyroid hormone production is too high, with a corresponding fall in TSH. Primary hypothyroidism occurs when insufficient amounts of thyroid hormone are available for uptake by the tissues and TSH subsequently rises (Kaplan and Pesce 2010).

Abnormal TSH levels will prompt further testing of freeT4 and potentially freeT3 to elucidate the underlying

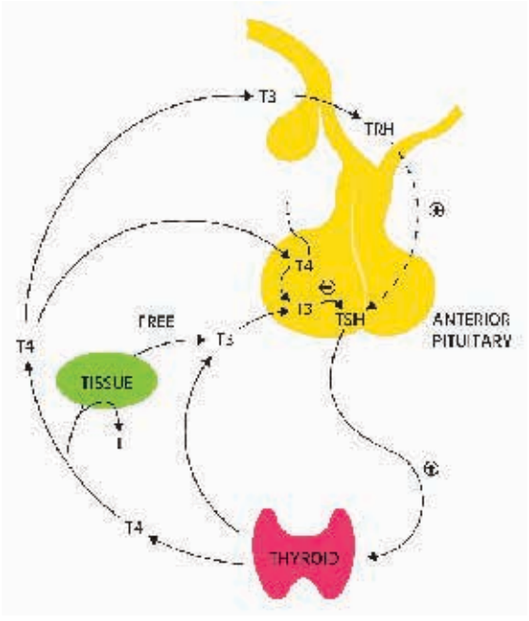

Figure 1. *Control of thyroid hormone secretion (TRH = thyrotropin-releasing hormone, TSH = thyroid stimulating hormone, T4 = thyroxine, T3 = triiodothyronine). Hypothalamic release of TRH acts on the anterior pituitary to control synthesis and release of TSH. TSH controls thyroid cell growth and hormone production by binding to a specific TSH receptor on the basolateral cell membrane of each thyroid cell. Circulating levels of T3 and T4 feedback to the anterior pituitary, with T3 also able to feedback directly to the hypothalamus (adapted from Gardner et al 2018).*

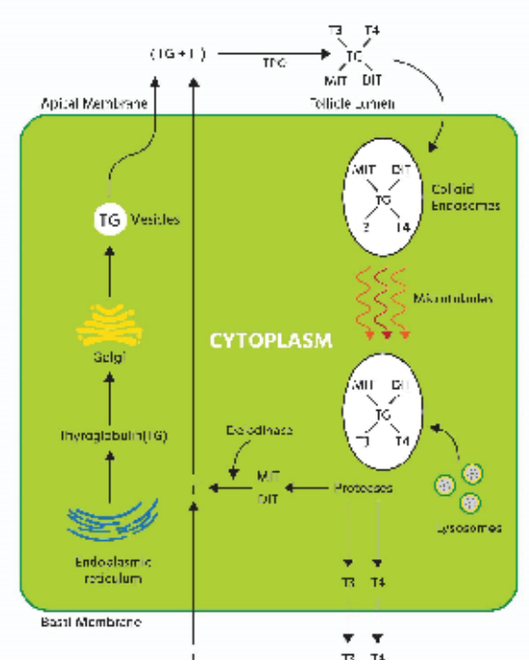

Figure 2. *Thyroid hormone production by the follicular cells (adapted from Koeppen et al 2008). (TG= thyroglobulin, T4 = thyroxine, T3 = triiodothyronine, MIT = 3-monoiodotyrosine, DIT = 3,5-diiodotyrosine) TG is a glycoprotein that serves as preformed matrix in which reactive iodine can attach to from MIT and DIT. Enzymatic coupling of MIT and DIT results in the formation of T3 and T4, which is released into the circulation (Kaplan and Pesce 2010).*

cause. The most common cause of hypothyroidism and hyperthyroidism is autoimmunity. Thyroid antibodies can be detected in autoimmune thyroid disease, including Hashimoto's (hypothyroidism) and Grave's disease (hyperthyroidism) and in patients without thyroid dysfunction (Frohlich and Wahl 2017). The diagnosis of these diseases is aided by identifying antibodies specific to components of the thyroid gland. Antibodies can most commonly be formed against TSH-receptor (TRAb), thyroid peroxidase (TPOAb) and thyroglobulin (TgAb) (Frohlich and Wahl 2017).

The foetus is dependent on the mother for placental transfer of thyroid hormones until the time it begins to synthesise its own at 18-20 weeks gestation (Gardner *et al* 2018). Maternal T3 does not cross the placenta so deiodination of maternal T4 by the foetus results in foetal production of T3. Consequently during pregnancy there is an increase in demand for maternal thyroid hormone production leading to an increase production of up to 50% (Forehan 2012).

ranges for TSH, to enable accurate screening for thyroid disease in pregnancy, will be explored.

Physiology of thyroid hormone regulation in pregnancy

A woman in the initial six weeks of pregnancy is physiologically indifferent to a normal adult female in terms of thyroid function (Rosario *et al* 2016). After the sixth week the physiological changes that occur are well documented, and non-contentious (Giacobbe *et al* 2015).

One of the first hormonal changes in normal pregnancy is the secretion of human chorionic gonadotropin (hCG) by the early placenta. hCG maintains the corpus luteum in the ovary to produce oestrogen and progesterone until the placenta can maintain the secretion (Figure 3).

hCG and TSH are both glycoprotein hormones and share a common alpha-subunit. Their beta-subunits share significant homology (Korevaar *et al* 2017). As hCG levels begin to rise in the first trimester, the molecule can bind to

***Table 1**. Thyroid hormones circulate in the blood in association with binding proteins (adapted from Kaplan and Pesce 2010)*

Binding protein	% T4 bound	Amount T3 bound
Thyroxine-binding globulin	60-75%	Majority
Transthyretin (prealbumin, TBPA)	15-30%	Negligibile
Albumin	10%	Small amount
Free hormone	0.02%	0.3%

The placenta contains iodothyronine deiodinase type III (DIO3). DIO3 inactivates thyroid hormones via inner ring deiodination of T4 to reverse T3 (rT3) and T3 to 3,3'-diiothyronine (3.3'-T2). Therefore the placenta plays a pivotal role in the regulation of thyroid hormone in the foetus (Laurberg *et al* 2013).

Both overt hypothyroidism and overt hyperthyroidism are associated with adverse pregnancy outcomes and neonatal outcomes, including neurological impairment (Carney *et al* 2014) (Forehan 2012). Hence the recognition of thyroid disease in pregnancy has implications for both maternal and neonatal well-being.

This review will discuss the normal physiology of thyroid hormone regulation in pregnancy, and the consequences of maternal thyroidal disease to the mother and baby. The current recommendations for screening for thyroid disease in pregnancy in Australia and internationally will be summarised and the use of trimester specific reference

the TSH receptor of thyroid follicular cells and stimulates the gland (Akarsu *et al* 2016). As a result hCG has weak thyrotrophic activity resulting in increased release of thyroid hormones and as hCG levels rise during pregnancy, there is a corresponding decline in TSH (Forehan 2012). The level of hCG in twin and multiple pregnancies is known to be higher and may produce more profound suppression of TSH (Ashoor *et al* 2013).

After the hCG peak, levels begin to decline and by the second trimester hCG (and TBG) are in plateau, leading to a more stable and reliable time for diagnosis of thyroid dysfunction (Qian *et al* 2013). TSH then slowly returns to normal after term (Akarsu *et al* 2016).

Thyroid TPOAb positivity is associated with a severely impaired thyroid response to hCG stimulation (Korevaar *et al* 2017). This suggests that impaired response to hCG stimulation may be how thyroid autoimmunity increases the risk of thyroid dysfunction during pregnancy (Korevaar *et al* 2017). The maternal iodide pool is reduced during

Table 2. *Prevalence of thyroid disease in Australia (adapted from Walsh 2016)*

Prevalence of Thyroid Disease in Australia		
Condition	**Definition**	**Prevalence**
Thyroid autoimmunity	Positive TPOAb or TgAb	12%
Subclinical hypothyroidism	Increased TSH, normal free T4	5%
Overt hypothyroidism	Increased TSH, low free T4	0.5%
Subclinical hyperthyroidism	Decreased TSH, normal free T4 and free T3	0.3%
Overt hyperthyroidism	Decreased TSH, elevated free T4 +/or free T3	0.3%
Palpable thyroid nodules		Approximately 5%
Thyroid nodules at ultrasound		Increases with age, up to 70% of the elderly

T3 = triiodothyronine, T4 = thyroxine, TgAb = thyroglobulin antibodies, TPOAb = thyroid peroxidase antibodies, TSH = thyroid stimulating hormone

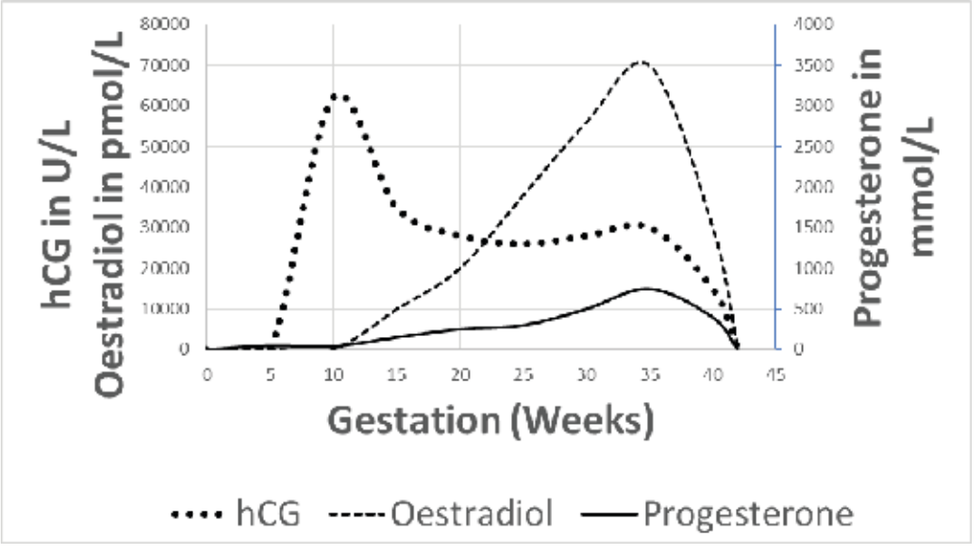

Figure 3. *Human Chorionic Gonadotropin (hCG), oestradiol and progesterone secretion during pregnancy. hCG rises rapidly in the first trimester reaching a peak at 8-9 weeks, it then falls and plateaus during the second and third trimesters (adapted from Ogueh et al 2011)*

pregnancy due to increased uptake for the synthesis of thyroxine in the maternal thyroid gland, increased renal clearance of iodide, and transfer of iodide from the mother to the foetus (Giacobbe *et al* 2015).

The effect of pregnancy on thyroid binding globulin (TBG)

Increased levels of placental oestrogens during pregnancy results in a marked increase in TBG. This begins during early gestation and reaches a plateau mid-gestation and is maintained at a higher level until delivery (Giacobbe *et al* 2015). The mechanism for the increase is two-fold, with an increased rate of production of TBG by hepatocytes and a concomitant reduced clearance of TBG from plasma. This expansion of the TBG extracellular pool leads to an progressive increase in the bound hormone fraction with an associated decrease in freeT3 and freeT4 (Giacobbe *et al* 2015).

The effect of pregnancy on thyroid stimulating hormone

TSH is a heterodimeric 28-kDa-glycoprotein released from the anteromedial pituitary (Estrada *et al* 2014). Its synthesis is controlled by thyrotropin releasing hormone secreted from the hypothalamus. Mature TSH contains both an alpha- and beta- subunit, and appropriate glycosylation is required to maintain TSH bioactivity (Estrada *et al* 2014).

Loss of glycosylation leads to loss of TSH bioactivity, while a higher rate of glycosylation reduces the TSH clearance rate from circulation, and leads to changes in hepatic and renal clearance. This natural variation in TSH glycosylation does not appear to change significantly during pregnancy (McNeil and Stanford 2015). As discussed previously, the most profound effects of pregnancy on TSH are the actions of increasing levels of hCG (tending to lower TSH), increased production of TBG and changes to the maternal iodide pool.

Thyroid disease in pregnancy

Thyroid disease can have negative effects on both the mother and the foetus and is often associated with adverse maternal or foetal outcomes such as increased incidence of abortion, gestational hypertension, placental abruption, post-partum haemorrhage, pre-term delivery, neonatal brain damage, mental retardation (Giacobbe *et al* 2015), and low birth weight (Medici *et al* 2015). A recent review (Medici *et al* 2015) found that pregnant women with a TSH greater than the population-based reference interval had an increased risk of premature delivery and intrauterine growth retardation. In contrast, pregnant women with TSH lower than this reference interval had an increased risk of hypertensive disorders (Medici *et al* 2015).

In the embryo, thyroid hormones are critical for early brain development, somatic growth and bone maturation (Springer *et al* 2017). The specific clinical consequences of lack of thyroid hormones depends on the gestational age of the foetus (Springer *et al* 2017). Thyroid hormone is supplied to foetal tissue via two sources, the developing foetal thyroid gland and the maternal thyroid gland, both of which are dependent on adequate iodine intake (Springer *et al* 2017). Maternal T4 is important in the first half of the pregnancy until the foetal thyroid can produce sufficient hormone (Springer *et al* 2017). Effects of thyroid disease in pregnancy can be partitioned into preconception, pregnancy and post-partum, as outlined in Table 3 (Carney *et al* 2014).

Pregnant women may have pre-existing thyroid disease, while others may develop it during pregnancy. The prevalence of thyroid disease in the normal Australian population is outlined in Table 2. Women with pre-existing thyroid conditions should be counselled on the importance of being euthyroid prior to conception, and also obtain advice on pregnancy-safe medications for the treatment of their disorder (Carney *et al* 2014).

Who is at risk?

Women at high risk of thyroid disease in pregnancy that may require testing include those with:

- History of thyroid dysfunction and/or thyroid surgery;
- Family history of thyroid dysfunction/goitre;
- Women with anti-thyroid antibodies with symptoms or clinical signs of hypothyroidism;
- Type 1 diabetes or other autoimmune disorders;
- History of miscarriage or pre-term delivery;
- Infertility;
- Prior head or neck irradiation;
- Morbid obesity, or pre-pregnancy BMI >30;
- Treatment with lithium or amiodarone (past six weeks);
- Exposure to iodinated radiological contrast agents;
- From an area of known moderate to severe iodine deficiency;
- History of post-partum thyroid dysfunction;
- Previous delivery of an infant with thyroid disease;
- TPOAb positivity.

Current recommendations for screening for thyroid disease in pregnancy

In 2014, prior to the release of the 2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum (Alexander *et al* 2017), Amouzegar and colleagues (2014) conducted a comparison of the American

Table 3. *Adverse outcomes associated with thyroid disease in pregnancy (adapted from Carneyet al 2014)*

Condition	Preconception	Pregnancy	Postpartum
Overt hyperthyroidism	Congenital malformations	Maternal: heart failure, placental abruption, preeclampsia, preterm delivery Foetal: goitre, intrauterine growth restriction, small for gestational age, still birth, thyroid dysfunction	
Subclinical hyperthyroidism		None	
Overt hypothyroidism	Decreased fertility, increased miscarriage	Anaemia, foetal neurocognitive deficits, low birth weight, miscarriage, placental abruption, preeclampsia, preterm delivery	Maternal thyroid dysfunction, haemorrhage
Subclinical hypothyroidism	Effects similar to overt hypothyroidism but less documentation exists		

Thyroid Association (ATA) (2011) and the Endocrine Society (ES) of America guidelines (2012). At that stage neither guidelines recommended universal screening of pregnant women and both supported case-based screening, however they were in agreement on whom the high-risk groups were. For high risk women, the ES recommended testing by the ninth week or at their first antenatal visit and the ATA agreed that TSH should be performed early in pregnancy in women at high risk of overt hypothyroidism (Amouzegar *et al* 2014). The ATA stated there was insufficient evidence for preconception testing for women at high risk of hypothyroidism. It still remains controversial whether or not to universally screen for thyroid disease before or during pregnancy.

The 2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum states “For universal screening to be recommended, any index condition must be prevalent, associated with adverse health outcomes and treatable. Furthermore, effective therapy must exist but also be practical and effectively deliverable. Finally, screening must be cost effective" (Alexander *et al* 2017). The recommendations made include:

- insufficient evidence to recommend for or against universal screening of TSH in early pregnancy, and
- all pregnant women should be verbally screened at the initial visit for the risk factors for thyroid disease (Alexander et al 2017).

In Australia, universal screening is not supported by the Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) (RANZCOG 2015, Forehan 2012). A review of the RANZCOG guidelines was due in 2018. Table 4 summarises the recommendations from each of the guidelines and shows that similar practice recommendations largely exist between each of these organisations. There is growing support in the literature for universal screening, and the evidence for both universal and case-based screening during pregnancy will now be examined. Consideration should be given to preconception screening of high risk women, although this requires further evaluation (Forehan 2012).

Case-based screening

Case-based screening is screening targeted at pregnant women who fall in to the high risk categories listed previously and this practice is widely used and supported by the current guidelines. However an increasing number of studies are finding that case-based screening is missing anywhere from 5% - 55% of pregnant women with thyroid dysfunction, resulting in untreated thyroid disease and potential risk to both the mother and the foetus (Forehan 2012; Jouyandeh *et al* 2015; Dave, Maru, and Tripathi 2014; Krassas *et al* 2015; Nazarpour *et al* 2016; Qian *et al* 2013).

Additionally one group found that targeted screening is only effective for those women with overt hypothyroidism, not those with subclinical hypothyroidism (Yang *et al* 2014), which is more common in Australia (Walsh 2016). Interestingly, Qian and associates (2013) support the screening of pregnant women in the second trimester when levels of TBG and hCG are in plateau, and the diagnosis of thyroid dysfunction is more stable and reliable (Qian *et al* 2013). Consideration must be taken as to what effect later diagnosis of thyroid dysfunction would have on the mother and the foetus. Qian *et al* (2013) did not analyse the results of the pregnancies, nor compare them with pregnancy outcomes of pregnant women screened in the first trimester and this makes their statement supporting second trimester screening flawed (Qian *et al* 2013).

Universal screening

Despite multiple guidelines stating there is insufficient evidence to support the use of TSH to screen all pregnant women for thyroid dysfunction, published studies from a variety of countries (retrospective and prospective) and

Table 4. *Recommendations of thyroid disease screening on preconception and pregnancy (ATA = American Thyroid Association, ES = Endocrine Society, ETA = European Thyroid Association, RANZCOG = Royal Australian and New Zealand College of Obstetricians and Gynaecologists).*

	ATA (2017)	ES (2012)	ETA (2014)	RANZCOG (2015)
SCH arising before conception or during gestation should be treated	YES recommended for TPOAb positive women with TSH > TSRR. May be considered in TPOAb negative women with TSH >10.0	YES in women with SCH who are TPOAb positive	YES	
Should overt hypothyroidism be treated in pregnancy	YES	YES	YES	Consensus based recommendation – YES
Is targeted based testing for hypothyroidism recommended in pregnancy	YES – based on clinical evaluation and risk factor identification	YES – based on history, medical examination or prior test results.	YES – even though it will miss a large percentage of women	Consensus based recommendation – YES
Is there sufficient evidence to support universal screening for hypothyroidism in pregnancy	NO – insufficient evidence	NO – insufficient evidence/evidence of poor quality. Members split between universal screening and aggressive case base finding.	NO Evidence is equivocal, but must be reassessed when new high-quality evidence is available	Consensus based recommendation - NO
Is screening for thyroid dysfunction prior to pregnancy recommended	NO – insufficient evidence	NO	NO	
Trimester specific TSH references ranges should be established in each antenatal hospital setting	YES	Mentioned in text but not recommendations.	YES	YES
If trimester specific TSH ranges are not available, the following upper limits are recommended	If TSRR unavailable 1. Ranges from similar populations and TSH platforms should be used (transferrance) 2. If not (1) then URL of ~ 4.0mU/L	>2.5 mIU/L with treatment aimed bring TSH below 2.5	1st trimester 2.5mU/l 2nd trimester 3.0 mU/l 3rd trimester 3.5 mU/l	TSH 1st trimester 0.1-2.5 mIU/L 2nd trimester 0.2-3.0 3rd trimester 0.3-3.0

systematic reviews are showing increasing evidence in support of universal screening (Dave *et al* 2014; Jouyandeh *et al* 2015; Krassas *et al* 2015; Nazarpour *et al* 2016; Qian *et al* 2013; Sastre-Marcos *et al* 2015; Springer *et al* 2017; Stagnaro-Green 2017; Yang *et al* 2014; Zornitzki *et al* 2014; Preda 2014). Springer *et al* (2017) believe the argument for universal screening is compelling – thyroid dysfunction is common in pregnancy, easy to detect and has an effective treatment that is safe and inexpensive. With targeted case-based screening missing one third to one half of women with thyroid disorders, more specialists are favouring universal screening. This group found universal screening met all the demands of Wilson and Jungner's principles screening for disease (Springer *et al* 2017).

The first antenatal visit in many areas of the world occurs in the first trimester and offers the opportunity to test TSH early in pregnancy. Given the high rate of missed diagnosis from targeted case-based screening, it would seem that a universal screening program would avoid potentially adverse outcomes to the mother and child.

If universal screening is not employed, all pregnant women can be verbally screened at the first antenatal visit, to assess their risk of thyroid disease via a patient questionnaire - an example of which is seen in Figure 4.

Trimester specific reference ranges for TSH

Thyroid function within a non-pregnant population can vary due to a number of factors including ethnicity, iodine status and body mass index (BMI) (Kumar *et al* 2017; Pop *et al* 2013). The physiological changes to thyroid function in pregnancy only adds to the difficulty in interpreting the results of thyroid function testing.

Patient Questionnaire	
Age/Date of Birth	
Height	
Weight	
Calculated BMI =	
Estimated due date: Date of last menstrual period (LMP)? Date by early gestation ultrasound?	
What is your ethnic background?	
Do you smoke cigarettes or other tobacco products?	
Do you consume iodised food products?	
Do you have a history of thyroid disease?	
Do you have a family history of thyroid disease?	
Are there any inherited disorders?	
Have you been diagnosed as having goitre?	
Have you been diagnosed with any type of autoimmune disease?	
Do you suffer from any type of systemic disease (ie something that affects more than one part of the body, or the whole body)?	
Have you ever undergone head/neck irradiation?	
Have you been diagnosed with diabetes – gestational or other?	
Have you suffered recurrent miscarriages?	
Do you, or have you suffered from hypertension (high blood pressure), pre-eclampsia or hyper-emesis gravidarum?	
What regular medications are you taking (prescribed or other)?	
Was this pregnancy a result of assisted reproduction?	
Has this, or previous pregnancies, been diagnosed with foetal abnormality, or small for gestational age?	
Have you ever tested positive for thyroid antibodies? Do you consent to being tested for common thyroid antibodies?	

Figure 4. *Example of a patient questionnaire*

Regardless of which screening program is employed, there is universal support in the literature for the use of trimester-specific TSH reference ranges to detect the presence of thyroid disease in pregnancy (Akarsu *et al* 2016; Alexander *et al* 2017; Bliddal *et al* 2014; Elahi and Hussain 2013; Gilbert *et al* 2008; Kim *et al* 2015; Maji *et al* 2014; Moon *et al* 2015; Patal *et al* 2016; Rajput *et al* 2016; Sekhri *et al* 2016; Shen *et al* 2014; Xing *et al* 2016; Kostecka-Matyja *et al* 2017; Dieguez *et al* 2016).

Discussion

Although agreement on the approach to screening for thyroid disease in pregnancy has not been reached, the literature is in consensus for the need for trimester specific reference intervals for TSH if we are to correctly classify thyroid dysfunction in pregnancy.

General TSH reference intervals from different methods and reagent manufacturers vary, as they have been established using pooled sera from males and non-pregnant females and employ a range of different antibodies in their detection systems. They are therefore not applicable to the pregnant population. Even the trimester specific reference intervals now provided by many manufacturers will vary significantly depending of the reference population they were derived from.

It is clear that population, laboratory and platform specific reference intervals are the gold standard. However this is often a logistically unachievable option for many laboratories and alternatives to full reference interval studies need to be considered. Even carefully selected reference populations may not provide intervals that are applicable across all pregnant women in a multi-cultural population, or with varying body mass index and iodine intake.

The wide variety of assays used in Australia, together with a presumed mix of manufacturer and laboratory derived reference intervals and a multi-cultural population further highlights the difficulty of interpreting TSH levels in pregnancy. It is also emphasises the importance of using a single laboratory for monitoring TSH during therapy. This is especially important for an assay like TSH where numerous studies have shown significant variation between the available platforms. While efforts towards harmonisation are underway, the introduction of common reference intervals is still a time away in the non-pregnant as well as the pregnant populations.

Once trimester specific TSH intervals have been adopted, the next area of thyroid disease in pregnancy that needs further evidence is the type of screening, if any, that should be used. Currently the international guidelines only support case-based screening in those patients considered to be at high risk of thyroid disease. However, it has been repeatedly shown that targeted case-based screening will miss one third to one half of women with thyroid disorders in pregnancy.

Conclusion

There is growing momentum in the recent literature to support the introduction of universal screening given that thyroid dysfunction is common in pregnancy and easy to detect with an effective treatment that is safe and inexpensive. The use of appropriately determined trimester specific reference intervals will improve detection and classification of thyroid disease in pregnancy. An expanded evidence base will provide the data required to support universal screening and further advance how we as diagnostic laboratories support the management of thyroid disease in pregnancy.

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ORIGINAL ARTICLE

A comparison of a biochemical test and gene amplification for identification of *Mycobacterium tuberculosis* Complex from pulmonary isolates in Surabaya Indonesia

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Abstract

Mycobacterium tuberculosis Complex (MTBC) is a primary cause of human pulmonary infection. The objective of this research was to study compatibility of the polymerase chain reaction (PCR) method using specific DNA sequences in the 16S rRNA gene region for MTBC identification compared to conventional biochemical tests. Clinical isolates of *Mycobacterium tuberculosis* were collected from patients with pulmonary tuberculosis in Dr. Soetomo Hospital Surabaya, Indonesia. Samples were collected randomly from January to July 2016. Samples were then assayed using a niacin accumulation test and PCR method having a target region of 1500 bp of the 16S rRNA gene. Eighty-seven *Mycobacterium* clinical isolates were identified by analyzing the 16S rRNA gene for MTBC. Eighty-three isolates were positively identified as MTBC, indicated by 1500 bp band. Compared to conventional methods, PCR method was found to have 100% concordance between the biochemical and PCR test. Specific DNA region at 1500 bp of 16S rRNA gene from *Mycobacterium tuberculosis* Complex was found to be a rapid and accurate method of identification.

Keywords: 16S rRNA gene, Mycobacterium tuberculosis *Complex, pulmonary tuberculosis isolates, Surabaya-Indonesia*

Introduction

One infectious disease requiring a surveillance system is tuberculosis (TB). This disease is still considered as a global health challenge with a high mortality and morbidity. In 2015, Indonesia was ranked as the country having the second highest prevalence of TB (WHO 2015). In 2014 the WHO reported nearly 9.6 million new TB cases with 1.5 million mortalities (WHO 2015). In 2013 there were 9.6 million new TB cases with 1.5 million mortalities. In the Association of South East Asia Nations (ASEAN), Indonesia is the country with the highest incidence of TB, estimated at about one million cases, and a prevalence of about 1.6 million TB cases with one hundred thousand mortality cases in a population of 254 million (WHO, 2015). TB remains a significant global health problem for which rapid diagnosis is critical for both treatment and control (Kazumi and Mitarai 2012; Perez-Osorio *et al* 2011). East Java is the Indonesian province with the second highest prevalence of TB after West Java. Surabaya, with a population of 2,801,409, has the highest population density in East Java,

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with approximately 8,463.47/km^2 (Public Health Office of East Java, 2013).

Mycobacterium tuberculosis Complex (MTBC) is a major cause of human pulmonary infection in many countries. MTBC is a group of acid-fast, aerobic, slow-growing bacteria (Forrellad *et al* 2013). A group of organisms known as *Mycobacterium avium* Complex (MAC) found in soil and water also commonly infects immunocompromised patients (Gordin and Horsburgh Jr, 2015). Many other species referred to as atypical or nontuberculous mycobacteria (NTM) have also been previously associated with TB (Cloud *et al* 2002). In clinical TB cases, identification of infecting *Mycobacterium* species is important in determining patients' treatment (Bicmen *et al* 2011).

Conventional biochemical identification methods have been employed as standard diagnostic procedure in general laboratories for a considerable length of time because this method needs more colonies. However, using these methods might result in ambiguous identification due to species phenotypic variation and variable test conditions, for example among members of MTBC have same phenotypic appeared (Coscolla and Gagneux 2014). In addition, these types of phenotypic testing are time consuming (Kazumi and Mitarai 2012).

The gene encoding small subunit of rRNA is found to be highly conserved. It also has complex parts with some variations in DNA loci that might affect species or genus identification (Baker *et al* 2003). Typically, 16S rRNA is used for specific identification in phylogenetic analysis of standard strains and clinical isolates (Cloud *et al* 2002; Kazumi and Mitarai 2012).

The 16S rRNA gene was chosen because this gene has a hypervariable region which is tolerant to mutation; and the other side, 16S rRNA gene continues to be conserved (Clarridge III 2004). Amplification of nucleic acid by PCR method is used for microbial identification because of its reported high sensitivity (Muthukumar *et al* 2008). This study aimed to compare the suitability of MTBC identification using a novel primer set in a PCR method on the specific DNA target of 16S rRNA and a phenotypic conventional biochemical method. The new primers can be used to detect *Mycobacterium tuberculosis* included hypervariable and conserved region.

Materials and methods

Clinical isolates

From January to July 2016, 87 samples of clinical *Mycobacterium* isolates were collected randomly from pulmonary tuberculosis patients in Dr. Soetomo Hospital, Surabaya, Indonesia. This research was granted ethical clearance by the Ethical Committee in Health Research of Dr. Soetomo Hospital Surabaya (No. 124/ Panke. KKE/ II/ 2014).

Culture method

Mycobacterium isolates were cultured on Lowenstein-Jensen medium (Merck, Germany) in duplicate with the solid medium Middlebrook 7H10 (Sigma Aldrich, USA). Resulting *Mycobacterium* clinical isolates were screened based on phenotypic characteristics, specifically colony morphology and Ziehl-Neelsen staining. Colonies were then collected for conventional phenotypic using the niacin accumulation test (TB Niacin Test Strips BD, Ireland, UK). Clinical isolates were differentiated based on this phenotypic identification test as either *Mycobacterium tuberculosis* Complex or non-tuberculous mycobacteria (NTM).

DNA extraction and polymerase chain reaction (PCR) method

DNA extraction was next performed from the MTBC colony using Qiagen extraction kit (DNeasy® Cat. No. 69504). Extracted DNA was amplified using PCR (MJ MiniTM Thermal cycle BioRed). The primers used were Forward: 5'-AGA GTT TGA TCC TGG CTC AG-3' and Reverse: 5'-AAG GAG GTG ATC CAG CCG CA-3'. The primer pair was designed using the target of 1500 bp specific region on the 16S rRNA gene of MTBC using genetic program MTBC H37Rv ATCC 27294. Primer was added 1 µl to PCR Mix (KapaBiosystem® Ready Mix). Amplification was initiated with denaturation at 95°C for 10 seconds, followed by annealing at 54°C for 10 seconds, and extension at 72°C for 15 seconds, so the PCR process was just around one hour and the cycle of the PCR is 30x. The temperature used for PCR was optimized prior to testing samples.

Electrophoresis

PCR product was visualized using ultrapure agarose gel 2% (Invitrogen, USA), stained using ethidium bromide and electrophoresis results can be seen for visualization by UV transluminator. The positive control used was 16S rRNA gene of *Mycobacterium tuberculosis* H37Rv strains ATCC 27294, while the negative control used was PCR mix (KapaBiosystem® Ready Mix) without DNA addition. Positive amplification result was indicated by the presence of 1500 bp band (see Figure 1).

Results

Of the initial 87 cultures, the results showed that 83 clinical specimens of pulmonary tuberculosis culture were positively classified as *Mycobacterium* species. By using either PCR or a conventional phenotypic method of niacin accumulation test, 83 (95.4%) samples were positively identified as MTBC, while the other four samples were

identified as negative. The MTBC-positive samples results of PCR and niacin accumulation test were concordant (Table 1).

Discussion

A conventional biochemical phenotypic method has been used as a standard method for *Mycobacterium* species identification in many countries. However, not all clinical isolates are phenotypically identical to a "type strain", and conventional tests were occasionally found to be erroneous (Kazumi and Mitarai 2012). Additionally, a conventional identification method was time-consuming, as typically seen on dysgonic growth (Kazumi and Mitarai 2012).

As seen in the current study, a concordance was found between a PCR method and a niacin accumulation test of samples with positive result of MTBC identification. The similarity of test results between the two methods indicating positivity was 95.4%. Both methods successfully detected MTBC with the same results, but the PCR method was found to be easier to perform compared with niacin test. The PCR is not time consuming. Niacin poses a danger to the environment, because it is a chemical substance that can damage soil fertility, thus the management of niacin waste needs to be controlled. A niacin test also requires a high number of colonies, with a minimum of 50 colonies required for a valid result (Remel 2005). Phenotypic-based conventional tests also have several limitations because various expressions of microbe enzyme protein are affected by strain growth due to interaction with the environment of tissue injury or inflammation reaction (Palomino *et al* 2007).

Although culture method is the gold standard for tuberculosis detection using solid and liquid medium along with development of diagnostic tools for tuberculosis diagnosis (Asmar and Drancourt 2015), a more rapid detection method is also required. For tuberculosis detection and identification, a molecular method could provide rapid detection. Nevertheless, the gold standard method is also still needed for validity and determination of tuberculosis diagnosis (Kassaza *et al* 2014).

One drawback of a biochemical conventional method for MTBC identification is that it is based on phenotype. Thus, this study needed further validity tests by determining the gold standard method for TB detection and observation for diagnosis discrepancy caused by co-morbid or co-infection with NTM, due to NTM in culture possibly being detected as MTBC. The diagnosis is a significant factor for the local government to determine treatment for TB patients, because TB is a chronic infectious disease. Recent increases of infectious disease caused by *Mycobacterium tuberculosis* as well as atypical mycobacteria could be quickly detected by using alternative methods instead of conventional methods based on culture and biochemical methods. In principle, this study could be performed by applying DNA or RNA amplification techniques with specific target genes (Dobner *et al* 1996).

Gene coding for small ribosomal subunit 16S rRNA is commonly used in bacterial phylogenetic analysis due to its universal distribution and mutation occurring at a slow and constant rate. At species level, 16S rRNA is considered to be stable and specific. The number of the 16S rRNA genes involved depends on bacterial species; for example, in enterococci, it contains four to six operons (rrn). 16S rRNA generally varies between species or subspecies, but when multiple 16S rRNA genes of the same isolates are sequenced, they are mostly identical or show only minor differences (Ninet *et al* 1996; Srinivasan *et al* 2015).

The presence of multiple different copies of 16S rRNA gene has been demonstrated previously for numerous bacterial genera and species by analyzing genomic sequence of different bacteria (Baker *et al* 2003). Another clinical strain previously studied, *Nocardia farcinica*, was revealed to possess three copies of 16S rRNA operon (Conville and Witebsky 2007).

As much as 98% similarity of 16S rRNA sequence is commonly used for species or strains identification (Clarridge III 2004). Based on this fact, a positive result requires further sequencing to understand differences on

Table 1. *MTBC identified by PCR using the specific DNA region target of 16S rRNA genes and niacin accumulation test of clinical isolates from the sputum of pulmonary TB patients in Dr. Soetomo Hospital Surabaya, January-July 2016.*

Test	Total samples (N)	Sample Positive MTBC (%)	Sample Negative MTBC (%)
PCR method	87	83 (95.4)	4 (4.6)
Niacin accumulation test	87	83 (95.4)	4 (4.6)

species level. *Mycobacterium smegmatis* generally has two operon rrn, which is different from slow-growing groups such as *Mycobacterium tuberculosis* and *Mycobacterium avium*, which only have one operon each. In general, one rRNA partial sequence is associated with its species (Ninet *et al* 1996; Adekambi and Drancourt 2004). If nucleic acid sequence has less than 97% similarity with the whole sequence of the 16S rRNA gene, the specimen could be declared as a new species (Rogall *et al* 1990; Sharma and Patil 2011).

Limitations of MTBC identification using 16S rRNA gene arise from many factors. One of the problems was using the specific 16S rRNA gene region only as this biomarker might cause high variation that could deter detection of MTBC. Sequence analysis of 16S rRNA specific gene has been widely used to identify bacterial species and perform taxonomic studies. The specific region gene of 16S rRNA generally contains nine "hypervariable regions" demonstrating considerable sequence diversity among different bacterial species and mainly used for species identification (Van de Peer *et al* 1996). Amplification of 16S rRNA gene specific region contains mostly highly-varied hypervariable regions and this target might cause inaccurate detection. Using the whole sequence of 16S rRNA gene with both hypervariable and conserved regions could limit variation, and thus detection would be more accurate.

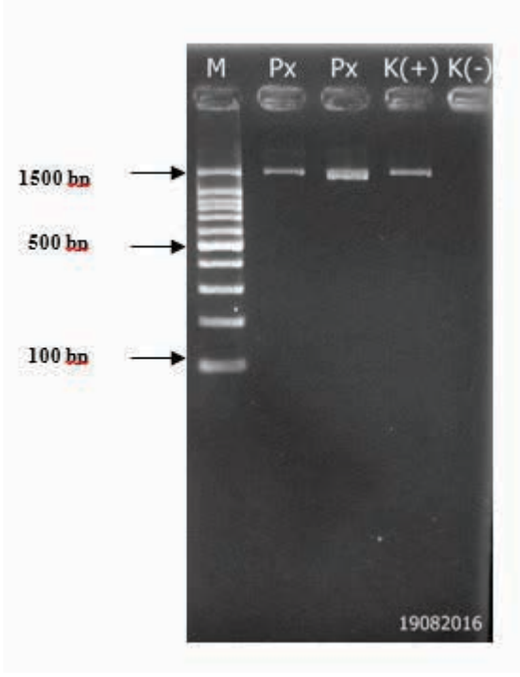

Figure 1. *PCR result using target of specific 16S rRNA gene region (Doc16S_Kul_Pub_1, 2016). Results are visualized in agarose gel electrophoresis. Specific amplicon produced was indicated by 1500 bp band. M = marker ladder; Px were positive samples; K+ = positive control; K- = negative control.*

Conclusion

Our study has indicated molecular identification using 16S rRNA gene as a rapid and comparable method to conventional testing, but we have suggested that it still has several limitations. Further study of molecular detection combined with other genes could potentially become a routine diagnostic method for *Mycobacterium* species.

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Ethical clearance

This research was granted ethical clearance by the Ethical Committee in Health Research of Dr. Soetomo Hospital Surabaya (No. 124/ Panke. KKE/ II/ 2014).

Conflict of interest

The authors declare no conflict of interest for the current study.

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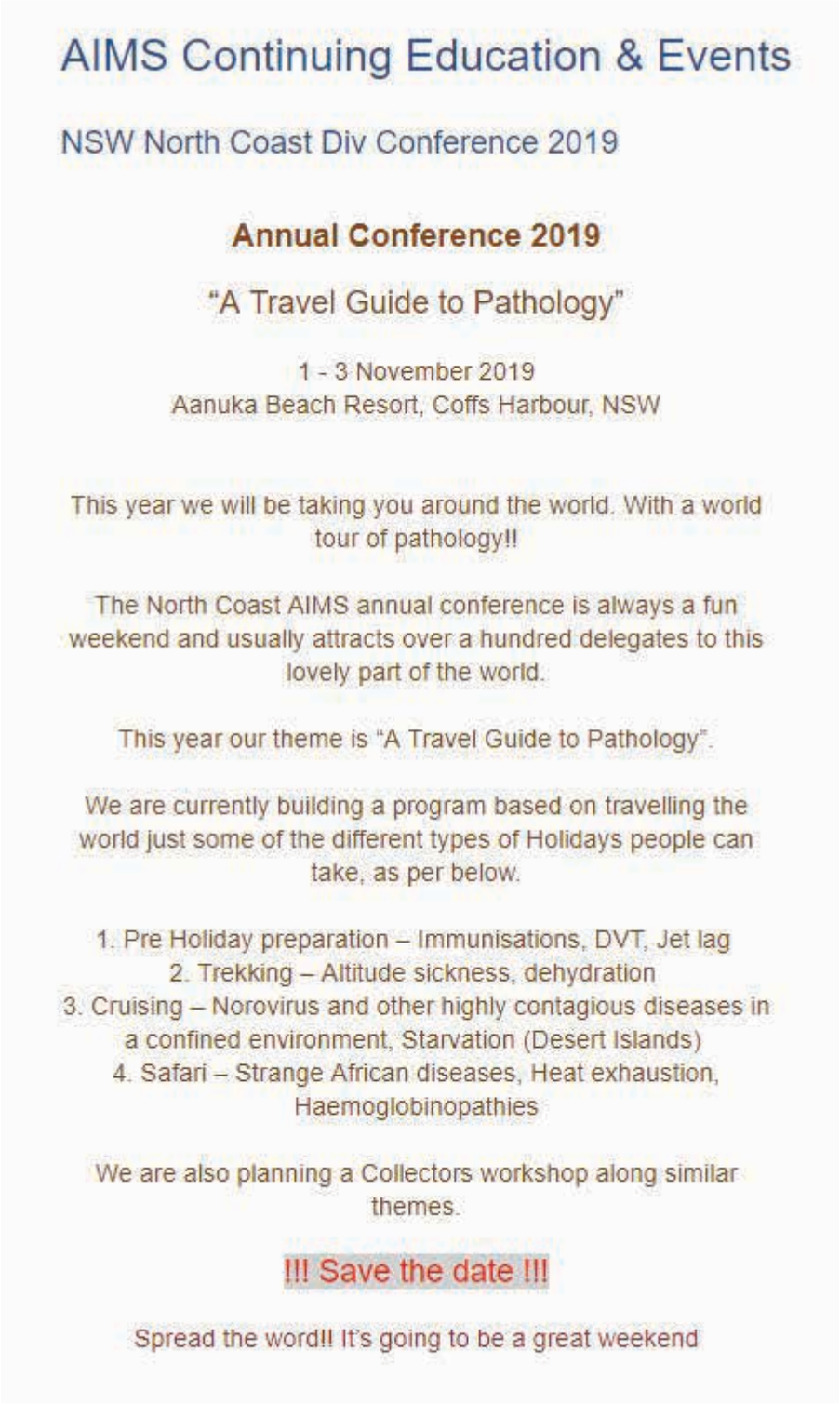

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The Australian Institute of Medical Scientists and the New Zealand Institute of Medical Laboratory Scientists is pleased to present their combined meeting, the South Pacific Congress, to be held at GCEC, Gold Coast, Queensland from 17-19 September 2019.

It is our great pleasure to invite you to join us all at this exciting event.

The conference, exhibition and associated social events provide a range of opportunities for continuing education and professional development, catching up with colleagues and friends, networking, and of course enjoying the beautiful sights of the Gold Coast. We expect over 400+ delegates to attend and look forward to your participation.

The theme for the congress is "New Wave Science". It is a three day conference which will attract delegates from Australia, New Zealand and other international countries. We have a comprehensive program which will appeal to all lab staff, ranging from phlebotomists, lab assistants, core lab scientists to specialised senior scientists and lab managers. The program has invited such high profile speakers such as the forensic anthropologist, Dr Donna MacGregor, international speakers from Ireland, Great Britain, Canada and NZ, cutting edge scientists such as Dr Ken Dutton-Register, Dr Maher Ghandi (named as one of the most influential researchers in Australia), and Dr Danielle Stanisic, along with many local and interstate experts. All the major disciplines will be covered, with a comprehensive pre-analytical 2 day program also offered.

Other highlights of the meeting include the Industry Exhibition, submitted oral papers and posters, networking functions, industry symposia, meet the experts breakfast sessions and of course the gala dinner.

A couple of speaker profiles

Professor Michael Reade is the Australian Defence Force (ADF) Chair of Military Medicine and Surgery and a member of the Burns, Trauma and Critical Care Research Centre at UQ. A specialist intensive care physician, anaesthetist and clinician-scientist, he leads a programme of research relevant to military trauma medicine and surgery, and guides the implementation of modern trauma care into ADF practice.

Professor Nathan Subramaniam's interests lie in the study of liver injury and understanding how the liver regulates iron homeostasis. Prof Subramaniam has many years of experience in the research of iron overload disorders and was instrumental in defining many genetic mutations associated with the iron overload disorder hereditary haemochromatosis. His basic science interests include defining the functional consequences of these disease-causing mutations and elucidating the molecules and mechanisms regulating iron homeostasis.

Dr Ken Dutton-Register. His research aims to understand the genetic events driving the growth of melanoma with the goal of using this information to improve survival outcomes for patients. He believes that a thorough understanding of the fundamental biology and core mechanisms of tumour development will result in effective, rationally designed therapeutic and early-intervention strategies.

Donna MacGregor is the Australian Army's only forensic anthropologist and was part of a team that brought home the remains of 33 Australians from the Terendak Military Cemetery in Malaysia, a burial site for soldiers who served in the Vietnam War. She is also a lecturer at QUT (Queensland University of Technology) teaching anatomy and forensic anthropology. Donna has also previously worked for 10 years with the Queensland Police Service.

Dr Danielle Stanisic is a Senior Research Fellow in the Institute for Glycomics, Griffith University and currently working on a malaria vaccine project. In a world first, her research group trialled the use of a whole parasite blood-stage malaria vaccine in human volunteers that has yielded safe and immunogenic outcomes.

President of AIMS

Ms Robyn Wells

South Pacific Congress 2019

Gold Coast

17-19 September 2019

Gold Coast Convention and Exhibition Centre

A P A C E

Australian Professional Acknowledgement of Continuing Education (APACE)

3 APACE credits per set of questions will be awarded if at least 8 out of 10 questions are answered correctly.
24 credits maximum per accreditation period claim.

Journal-based CPD No. 60
Page 1 of 1

Questions relating to the article *'Thyroid disease in pregnancy'* at page 2 of this issue.

1.	Thyroid hormone secretion is controlled via the hypothalamic-pituitary-thyroid axis.	True/False
2.	The thyroid gland sits in the anterior neck. It normally comprises two small lobes connected by an isthmus at the midline.	True/False
3.	The physiological effect of thyroid hormones is not to control cellular metabolism.	True/False
4.	The daily secretion of thyroid hormone includes 80-100μ of T4 and around 7μ of T3.	True/False
5.	Hyperthyroidism is a disease state where thyroid hormone production is very low, with a corresponding fall in TSH.	True/False
6.	Primary hypothyroidism occurs when insufficient amounts of thyroid hormone are available for uptake by the tissues and TSH subsequently rises (Kaplan and Pesce 2010).	True/False
7.	The most common cause of hypothyroidism and hyperthyroidism is autoimmunity.	True/False
8.	The foetus is dependent on the mother for placental transfer of thyroid hormones until the time it begins to synthesise its own at 22-24 weeks gestation.	True/False
9.	Both overt hypothyroidism and overt hyperthyroidism are associated with adverse pregnancy outcomes and neonatal outcomes, including neurological impairment (Carney, Quinlan, and West 2014) (Forehan 2012).	True/False
10.	One of the first hormonal changes in normal pregnancy is the secretion of human chorionic gonadotropin (hCG) by the early placenta.	True/False

Name: _____

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Journal-based CPD No. 61
Page 1 of 1

Questions relating to the article *'A comparison of a biochemical test and gene amplification for identification of* Mycobacterium tuberculosis Complex *from pulmonary isolates in Surabaya Indonesia'* at page 14 of this issue.

1.	Tuberculosis is no longer considered as a global health challenge with a high mortality and morbidity rate.	True/False
2.	In 2015, Indonesia was ranked as the country having the second highest prevalence of TB (WHO 2015).	True/False
3.	East Java is the Indonesian province with the second highest prevalence of TB after West Java.	True/False
4.	*Mycobacterium tuberculosis* Complex (MTBC) is not a major cause of human pulmonary infection in many countries.	True/False
5.	A group of organisms known as *Mycobacterium avium* Complex (MAC) found in soil and water also commonly infects immunocompromised patients (Gordin and Horsburgh Jr 2015).	True/False
6.	Amplification of nucleic acid by PCR method is used for microbial identification because of its reported high sensitivity (Muthukumar *et al* 2008).	True/False
7.	By using either PCR or a conventional phenotypic method of niacin accumulation test, 83 (95.4%) samples were negatively identified as MTBC, while the other 4 samples were identified as positive.	True/False
8.	A conventional biochemical phenotypic method has been used as a standard method for Mycobacterium species identification in many countries.	True/False
9.	A conventional identification method was time-consuming, as typically seen on dysgonic growth (Kazumi and Mitarai 2012).	True/False
10.	A niacin test requires a high number of colonies, with a minimum of 50 colonies required for a valid result (Remel 2005).	True/False

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AIMS Research Engagement Grant Scheme

Bursary and Grant Funding Opportunities



The AIMS Research Engagement Grant Scheme aspires to recognise, support and engage with research-oriented medical scientists through annual bursary and grant opportunities to encourage pathology-related research and CPD. For enquiries and expressions of interest please email contact@aims.org.au.

AIMS will be accepting applications after the 23rd of September 2019.

FUNDING OPPORTUNITIES	ELIGIBILITY	VALUE (AUD)
Scientific Communication Bursary	Graduate or final year student from a university offering an AIMS accredited program	2 x $500
Honours Research Bursary	Candidates enrolled at a university offering an AIMS accredited program	3 x $1000
MPhil or Master by Research Seeding Grant	Candidates enrolled at a university offering an AIMS accredited program	2 x $2,500 or 1 x $5,000
Doctor of Philosophy (PhD) Research Seeding Grant	Candidates enrolled at a university offering an AIMS accredited program	2 x $5,000 or 1 x $10,000
AIMS Fellowship (Stage 4) Bursary	Candidates enrolling in or undertaking Stage 4 of the AIMS Fellowship	3 x $2000
Clinical or Laboratory Research Grant	Medical scientists with an acceptable* degree	2 x $5,000 or 1 x $10,000
Education or Leadership & Management Research Grant	Medical scientists with an acceptable* degree	2 x $5,000 or 1 x $10,000

https://www.aims.org.au/cpd/research-engagement-grant-scheme#

https://www.aims.org.au/documents/item/1216

A case of promyelocytic leukaemia–variant (APL-V) in a fifty-six-year-old male

Gillian Rozenberg

South Eastern Sydney & Illawarra Area Health Service, Prince of Wales Hospital, New South Wales

A fifty-six-year-old male presented to ED with a severe headache.

Upon examination he was noted to have visual changes on a background of HIV, chronic kidney disease (CKD), hepatitis C, hypertension, gout, hypercholesterolaemia and depression.A Full Blood Count was performed with the following results:

Hb	111	RR 130-180 g/L
MCV	103.0	RR 80-100 fL
MCH	36.9	RR 26.5-33.0pg
WBC	33.25	RR 3.50-11.00 x 10^9/L
Platelet	16	RR 150-400 x 10^9/L

Neutrophil	3.0	%
Lymphocyte	6.0	%
Monocyte	0.0	%
Eosinophil	0.0	%
Basophil	0.0	%
Promyelocyte	90	%
Blast	1	%
Lymphocyte	2.00	RR 1.5-4.0 x 10^9/L
Neutrophil	1.00	RR 1.7-7.0 x 10^9/
Monocyte	0.00	RR 0.1-0.8 x 10^9/L
Eosinophil	0.00	RR 0.04-0.44 x 10^9/L
Basophil	0.00	RR 0.0-0.2 x 10^9/L

Blood film findings:

- Occasional NRBC
- Anisocytosis – Slight
- Macrocytes – Slight
- Polychromasia – Slight

Note the presence of abnormal promyelocytes.

Coagulation studies were immediately requested by the morphologist reviewing the blood film. The results were as follows:

Pt	15.7	RR 12.0-15.0 sec
INR	1.2	
APTT	24.9	RR 25.0-37.0 sec
Fibrinogen	4.3	RR 2.20-4.30 g/L
D Dimer	3.16	RR <0.5 mg/L

The coagulation results were mildly deranged.

The following tests were performed:

Flow Cytometry

Flow Cytometry was performed on the peripheral blood with the following results:

CD45+/HLA DR-/CD13+/CD14-/CD15-/CD33+/CD64+/CD34+/CD117+/MPO+

80% (of total cells) were blasts and promyelocytes identified with the above phenotype.

Consistent with APL hypogranular variant.

Cytogenetics and FISH studies were performed.

Cytogenetics / FISH

G-banded cytogenetic analysis revealed a translocation between the long arms of chromosomes 15 and 17.

FISH analysis using the Metasystems PML/RARA dual fusion probe showed fusion signals consistent with t(15;17) in 85% of cells examined.

A bone marrow aspirate and trephine were also performed.

Bone marrow

Erythropoiesis was markedly reduced.

Granulopoiesis showed a maturation arrest in the promyelocyte stage.

Megakaryopoiesis was markedly reduced with normal morphology.

There was an increase in blasts and promyelocytes (69% of the nucleated cells).

The blasts were medium sized with a high N/C ratio, basophilic cytoplasm and few fine azurophilic granules and Auer rods.

The promyelocytes were abnormal with bilobed, reinform shaped nuclei also containing few cytoplasmic granules and Auer rods. There was a maturation arrest in the promyelocyte stage.

Trephine

The trephine was densely packed with abnormal promyelocytes and blasts.

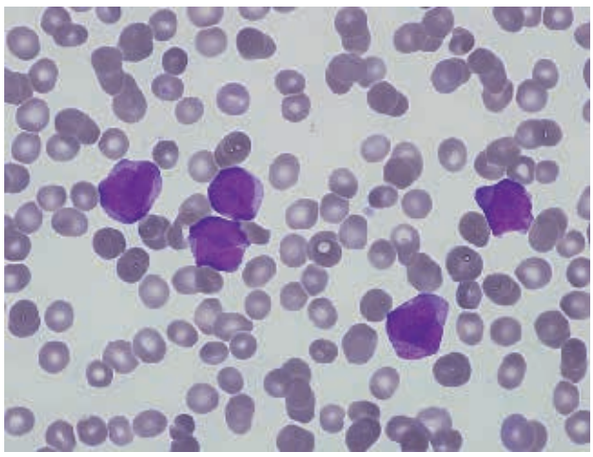

Figure 1. *Peripheral blood film showing the presence of bilobed abnormal promyelocytes.*

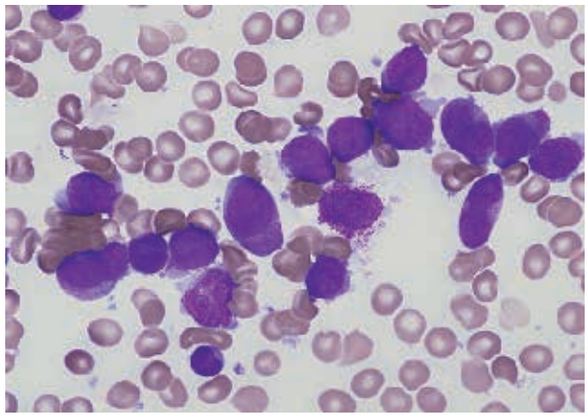

Figure 2. *Bone marrow showing an infiltrate of blast cells and abnormal promyelocytes.*

The findings are consistent with acute promyelocytic leukaemia - hypogranular variant.

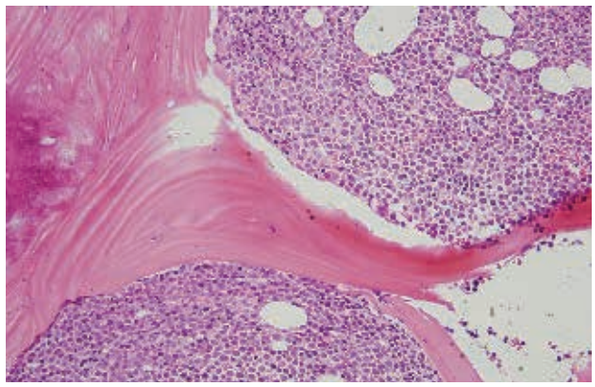

Figure 3. *Bone marrow trephine showing an infiltrate of blasts and abnormal promyelocytes with reinform and bilobed nuclei.*

References

Palmer L, Briggs C, Mcfadden S, Zini G, Burthem J, Rozenberg G Proytcheva M, Machin S J 2015. 'ICSH recommendations for the standardization of nomenclature and grading of peripheral blood cell morphological features'.

Moschcowitz E 1924. Hyaline thrombosis of the terminal arterioles and capillaries: a hit her to underscribe disease. *Proc NY Pathol Soc* 24:21-24.

THE EVENT

Pathology Horizons is an annual and open CPD conference organised by Cirdan to discuss what developments lie ahead in Pathology and what we can do to prepare or take advantage of these.

Why attend?

This unique event will give you the chance to learn about new technologies, procedural developments and lines of research that are driving the future of pathology. You will hear about the possible pros and cons of these developments, how people in supporting disciplines should be preparing for and perhaps directing these changes.

Pathology Horizons provides an opportunity for you to contribute your ideas, interact with innovators from around the world, expand your knowledge and make some new contacts.

CPD

Pathology Horizons has been approved by the CPD Certification Service, therefore enabling you to gain CPD points from attending. The conference has been approved by the New Zealand Institute of Medical Laboratory Scientists (NZIMLS) for 20 points. Th NZIMLS CPD approval code can be added to your certificate of attendance, if requested.

https://www.aims.org.au/services/non-aims-events/pathology-horizons

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The APACE (Australasian Professional Acknowledgement of Continuing Education) scheme is a voluntary programme that recognises continuing education, formal courses and a wide range of professional activities which contribute to your professional growth.

APACE offers an online user-friendly diary to record your professional activities. The APACE website features a step-by-step guide and our National Office staff are on hand to help. If you are an AIMS member, when you register for an AIMS event, your CPD points are automatically added to your diary.

APACE has been approved by the New Zealand Medical Laboratory Science Board as a re-certification programme for New Zealand Medical Laboratory Scientists and by the Royal College of Pathologists Australia (RCPA) as a continuing professional development recognition programme for Fellows of the Faculty of Science. APACE is also a great way to ensure compliance with NPAAC requirements for Medical Pathology Services.

AUSTRALIAN INSTITUTE
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BOOKS FOR REVIEW

Following is a list of books available for review by resource consultants and members of the Institute with particular expertise in the field. The reviewer is invited to retain the complimentary copy of the book once the review is received.

As per our agreement with the book publishing companies, complimentary books are submitted to the Institute provided that all reviews are published in the Australian Journal of Medical Science. These reviews must be of a high quality as buying decisions and the reputation of the book and author are important considerations.

Books not requested will be allocated at discretion of the Editors for the Australian Journal of Medical Science. Reviews should be 300 to 700 words depending on the volume of the book. Time limit for return of review is **six** weeks.

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1. **Bifidobacteria: Genomics & Molecular Aspects** edited by B. Mayo, & D. Van Sinderen. Caister Academic Press. xii + 260 pages.

2. **Medicine and Sport Science Volume 55: Cytokines, Growth Mediators & Physical Activity in Children during Puberty** edited by J. Jurimae, A.P. Hills & T. Jurimae. Karger. viii+178 pages.

3. **Digestive Diseases The Keys to IBD 2010: Treatment, Diagnosis & Pathophysiology.** Edited by G. Rogler & W. Sandborn. Karger. 188 pages.

4. **Else Kröner-Fresenuis Symposia Volume 1: Molecular Mechanisms of Adult Stem Cell Aging** edited by K.L. Rudolph. Karger. xii+108 pages**.**

5. **Endocrine Development Volume 24: Hormone Resistance and Hypersensitivity** edited by M. Maghnie, S. Loche, M. Cappa, L. Ghizzoni & R. Lorini. Karger. viii + 160 pages.

6. **Frontiers of Hormone Research Volume 41: Endocrine Tumor Syndromes and Their Genetics** edited by C.A Stratakis. Karger. xii + 187 pages.

7. **Frontiers of Hormone Research Volume 39: Kallmann Syndrome & Hypogonadotropic Hypogonadism** edited by R. Quinton. Karger. x+174 pages.

8. **Frontiers of Neurology & Neuroscience Volume 27: Neurological Disorders in Famous Artists: Part 3** edited by J Bogousslavsky, MG Hennerici, H Bäzner, C Bassetti. Karger. 240 pages.

9. **Generic: The Unbranding of Modern Medicine** by Jeremy A. Greene. John Hopkins University Press. 368 pages.

10. **Human Pathogenic Fungi: Molecular Biology and Pathogenic Mechanisms** edited by Derek J. Sullivan & Gary P. Moran, Caister Academic Press. x + 342 pages.

11. **Internal Medicine: A Doctor's Stories** by Terrence Holt. Black Inc. 273 pages.

12. **Intolerant Bodies: A Short History of Autoimmunity** by Warwick Anderson and & Ian R. Mackay. John Hopkins University Press. 250 pages.

13. **More Than Hot: A Short History of Fever** by Christopher Hamlin. John Hopkins University Press. 400 pages.

14. **Pediatric and Adolescent Medicine Volume 19: Metabolic Syndrome and Obsesity in Childhood and Adolescence** edited by W. Kiess, M. Wabitsch, C. Maffeis, A.M. Sharma. Karger. x + 202 pages.

15. **Phage Therapy - Current Research and Applications** edited by Jan Borysowski, Ryszard Miedzybrodzki & Andrzej Gorski. Caister Academic Press. 368 pages.

16. **Shigella: Molecular and Cellular Biology** edited by William D. Picking & Wendy L. Picking. Caister Academic Press. 280 pages

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AUSTRALIAN JOURNAL OF MEDICAL SCIENCE

Instructions to authors

The following instructions are based on the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals", also known as the Declaration of Vancouver, and on the *Australian Government Style manual: for authors, editors and printers*, 6th edition, 2002. URLs were correct on September 29th, 2008.

Manuscripts that do not fully comply with the following 'Instructions to Authors' may be returned for revision before they are considered for publication.

The *Australian Journal of Medical Science (AJMS)* will consider for publication any paper relevant to the field of Medical Science. Disciplines include blood banking, clinical biochemistry, haematology, histopathology, immunology, microbiology and molecular biology. Areas of general interest to medical laboratory scientists, including toxicology, epidemiology, public and community health, and professional and management issues will also be considered.

Papers published in the *AJMS* are in the form of:

- Review Articles
- Original Articles
- Brief Communications
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- Case Studies
- Letters to the Editor
- Book Reviews

Articles submitted for publication are understood to be offered only to the *AJMS* and those accepted become the property of the *AJMS*.

All individuals listed as authors must have made a substantial contribution to the conception and design of the study, the acquisition of data or the analysis and interpretation of data; the drafting of the article or revising it critically for important intellectual content; and final approval of the version to be published. The corresponding author must take responsibility for obtaining permission from all the authors for the submission of any version of the manuscript and for any changes in authorship.

When the manuscript is submitted the authors must disclose any potential conflict of interest and/or commercial support.

Requirements & preparation of manuscripts

General

Articles should be submitted in electronic format to programs@aims.org.au. If an article is too large to be submitted by email, it should be submitted on an or USB stick.

Number pages consecutively commencing with the title page.

Arrange the article in the following sequence:

- Title page
- Abstract and key words
- Main Text
- Acknowledgements
- References
- Tables - each table, complete with title and footnotes, on a separate page
- Legends for illustrations.

Authors should ensure that their manuscript communicates their ideas and concepts simply and clearly so that the article is easily read and understood. Authors are strongly recommended to refer to the recommendations on reporting standards as outlined in the statements and checklists of the CONSORT group (see: http://www.consort-statement.org/) and similar groups such as STARD (see: http://www.stard-statement.org/). The principles outlined in these standards may be used as general guidelines and not just as applied to clinical trials and diagnostic studies.

Title page

The title of the article should not exceed three lines (40 characters per line), including punctuation and spacing. All authors must be identified on the title page (e.g., William Smith, Susan Yeo, ...''). Where applicable, the title page should also include the name of the institution with which each author is affiliated and to which the work should be attributed. In the case of multiple authors, the name, postal address, email address, telephone and facsimile number of the author responsible for correspondence relating to the manuscript should be indicated.

Abstract & keywords

The abstract should be approximately 150 words and should make sense when read alone or in conjunction with the article. The abstract should be a concise overview that describes the important details of the article including the purpose of the study/investigation, basic procedures (study subjects/experimental animals/observational and analytic methods) and the results and principal conclusions. New and important aspects of the work and its implications may also be included. References should not be included.

Three to ten keywords may be listed. Authors are advised to comply with the terms from the Medical Subject Headings (MeSH) list from Index Medicus (see http://www.nlm.nih.gov/mesh/). Keywords should be given below the Abstract.

Text

The style of writing should conform to acceptable English usage. Do not use slang, medical jargon or unnecessary abbreviations. Accepted spelling is the first choice given in the latest edition of the Macquarie Dictionary.

Wherever possible, observational or experimental articles should be divided into sections headed:

- Introduction
- Materials and methods
- Results
- Discussion
- References

For other types of articles such as commentaries, reports and reviews, use an appropriate format or consult the Editors for guidance. Do not include a separate section for conclusions, these should be given in the discussion.

Introduction

Clearly state the purpose of the article leading the reader from the known to the unknown. Summarise the rationale for the study and state the question to be answered as appropriate. Give only strictly pertinent references, and do not review the subject extensively.

Materials & methods

Present the materials and methods in a logical sequence. Describe the selection of the observational or experimental subjects (patients or experimental animals, including controls) clearly. Notification of ethics approval must be given where relevant. Identify the methods, apparatus and procedures in sufficient detail to allow other workers to reproduce the results. Give references to established methods, including statistical methods. Adequately describe new or substantially modified methods. Identify precisely all drugs and chemicals used, including generic name(s), dosage(s), and route(s) of administration. Do not identify patients or hospitals without consent.

Results

Present the results in the same sequence as given in the Materials and methods; use tables and illustrations where these will help the reader understand the work being presented. Do not repeat in the text all the data in the tables or illustrations.

Discussion

Indicate the new and important aspects of the study and emphasise the conclusions that follow. Do not repeat in detail data given in the Results section and do not add new data. Include in the Discussion the implications of the findings and their limitations and compare the observations to other relevant studies. Recommendations may be included if appropriate. Link the conclusions with the goals of the study and answer the experimental question stated in the Introduction. However, avoid unqualified statements and conclusions not completely supported by your data. Avoid claiming priority and alluding to work that has not been completed. State new hypotheses when warranted, but clearly label them as such.

Acknowledgements

Acknowledge individuals who have made substantial contributions to the study including technical work and financial support. Authors are responsible for obtaining consent from all the individuals acknowledged by name as inclusion may be interpreted as an endorsement of the article's contents.

References

The AJMS uses a modified Harvard System (author-date system).

Throughout the body of the manuscript cite the author/s name and the publication year in parentheses as in the following examples:

(i) Research in this area (Jones 1999) ...

(ii) It has been successfully demonstrated that (Smith and Brown 1981; Auteur 1995; Scienziato *et al* 2007).

(iii) Following further investigation, Wetenschapper (2002 highlighted the difficulties inherent in...

Where there are three or more authors, acknowledge only the first author, e.g., (Smith *et al* 2007). For two authors the following style should be used: (Smith and Brown 2007).

The reference list should be in the format described below. Journal titles should be abbreviated in Index Medicus format (see: ftp://nlmpubs.nlm.nih.gov/online/journals/ljiweb.pdf) using standard abbreviations from the ISSN List of Title Word Abbreviations (see: http://www.issn.org/en/node/344) All authors should be given in the reference list.

Do not use abstracts as references. "Unpublished observations" and "personal communications" may not be used as references, although references to written, not verbal, communications may be cited (in parentheses) in the text. Include in the references manuscripts accepted but not yet published, designate the journal followed by "in press" (in parentheses). Information from manuscripts submitted but not yet accepted should be cited in the text as "unpublished observations" (in parentheses).

Examples of the correct form for references are given below:

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Stein MK, Downing RW, Rickels K 1978. Self-estimates in anxious and depressed outpatients treated with pharmacotherapy. *Psychol Rep* 43: 487-492.

Personal Author(s) of a book:

Osler AG 1976. *Complement: mechanisms and functions*. Englewood Cliffs: Prentice-Hall.

Editor, Compiler, Chairman as Author:

Rhodes AJ, Van Rooyen CE, comps. 1968. *Textbook of virology: for students and practitioners of medicine and the other health sciences*. 5th ed. Baltimore: Williams and Wilkins.

Chapter in Book:

Weinstein L, Swartz MM 1974. Pathogenic properties of invading microorganisms. In: Sodeman WA Jr, Sodeman WA, eds. *Pathologic physiology: mechanisms of disease*. Philadelphia: WB Saunders; 457-472.

Online documents:

National Center for Biotechnology Information. OMIM: online Mendelian inheritance in man. http://www.ncbi.nlm.nih.gov/omim. Accessed February 25, 2007.

Tables

Number tables consecutively with Arabic numerals and supply a brief title for each. Give each column a short or abbreviated heading. Place explanatory matter in footnotes, not in headings. Explain in footnotes all non-standard abbreviations used in each table.

For footnotes, use the following symbols in this sequence:

* † ‡ § ¶ ** ††

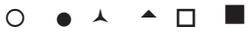
In preparing tables, consideration should be given to the page width of the Australian Journal of Medical Science. All tables should be prepared for publication vertically. In the text, cite each table in consecutive order, and mark in the margin of the text its approximate location.

If data from another published or unpublished source is used, written permission must be obtained and a copy must accompany the manuscript.

Illustrations

Colour illustrations may be submitted on a CD. Images should be scanned at a minimum of 300 dpi.

When plotting points, the following symbols are preferred:



In most instances, figures will be reduced to one column in width. All letters and numbers should be drawn to be at least 1.5 mm high after reduction, symbols at least 1.0 mm. Titles for illustrations belong in the legends for illustrations and not on the illustrations themselves.

Photomicrographs must have internal scale markers and the magnification must be stated. Symbols, arrows, or letters used in the photomicrographs should contrast with the background.

Cite each figure in the text in consecutive order, e.g., "Figure 1 illustrates …" or "… as shown (Figure 2)". If a figure has been published, acknowledge the original source and submit with the manuscript written permission from the copyright holder to reproduce the material. Permission is required, regardless of authorship or publisher, except for documents in the public domain.

Legends for illustrations

When symbols, arrows, numbers, or letters are used to identify parts of illustrations, identify and explain each one in the legends. The figure legend must contain a boldface (a) name ("Figure" + arabic figure number) and (b) substantive title.

Abbreviations

Use only standard abbreviations (see list of commonly used abbreviations).

Avoid abbreviations in the title. The full term for which an abbreviation stands must precede its first use in the text unless it is a standard abbreviation for a unit of measurement.

Report measurements in the units in which the measurements were made. In most countries the International System of Units (SI) is standard.

Commonly used abbreviations

Abbreviation or Symbol	Standard Units of Measurement
g	gram
g	gravity
Hz	hertz
h	hour
IU	international unit
K	kelvin
kg	kilogram
L	liter, litre
m	meter, metre
min	min
M	molar
mL	millilitre
mol	mole
N	newton
nm	nanometre
p	probability
rpm	revolutions per min
s	second
wk	week
yr	year

Additional information

The following are useful sources of information. The first two publications are used by the AJMS as standard references.

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Matthews JR, Matthews RW. *Successful scientific writing: a step-by-step guide for the biological and medical sciences*. 3rd ed. Cambridge, Cambridge University Press, 2007 [Also available in eBook format.]

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