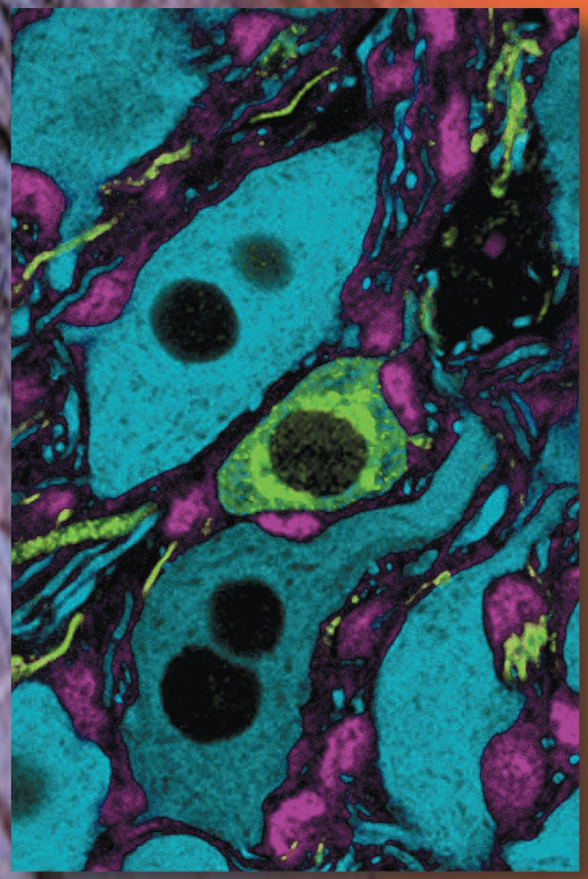
AUGUST 2020 VOL. 41 No. 3 Pages 85 - 119 AUSTRALIAN JOURNAL OF MEDICAL SCIENCE

August 2020
Vol. 41
No. 3

AUSTRALIAN JOURNAL OF

Medical Science

2020



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The Australian Journal of Medical Science is the official publication of the Australian Institute of Medical Scientists.

Circulation 2000 per issue. The Journal is circulated to members in pathology laboratories, universities and research institutes throughout Australia and overseas.

Annual subscription rates are available from the AIMS National Office.

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Abstraction of the Australian Journal of Medical Science is through the following serial catalogue listings: Australasian Medical Index, Chemical Abstracts, and EMBASE/Excerpta Medica.

The Australian Journal of Medical Science is listed on the Register of Refereed Journals by the Australian Commonwealth Department of Education Employment and Workplace Relations.

ISSN 1038-1643

Printed by Westminster Eagle Eye Printing, PO Box 161, Paddington Qld 4064.

Design Cover and layout design by Kim Brown, 23 Denman St Exeter SA 5019 kimbo@internode.on.net. Cover photograph courtesy of Prof Ian Gibbins, Flinders Medical Centre, Adelaide.

CONTENTS

August 2020 Vol. 41 No. 3

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

A rapid, reliable and inexpensive PCR assay for detecting *bla*-OXA-23 in clinical isolates of *Acinetobacter baumannii* using SYBR Green technology

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Abstract

Carbapenem-resistance amongst *Acinetobacter* spp. is commonly associated with the acquisition of the *bla*OXA-23 gene. Rapid laboratory detection is reliant on PCR assays as phenotypic expression of OXA-encoding enzymes is often weak in hydrolysis methods. A novel molecular approach to the detection of this gene in *Acinteobacter* spp. was evaluated using a SYBR Green-PCR format developed by McIver *et al* (2017) which circumvents traditional extraction methods and utilizes 75% less reagents. Using this method, 11 characterised clinical isolates of *Acinetobacter baumannii* known to carry the *bla*OXA-23 gene rapidly amplified a product with a melting temperature mean of 77.3°C (95% CI = 77.0 – 77.5°C). This was clearly discernible from a diverse range of 89 isolates of *Acinetobacter* spp. and unrelated strains including a proportion carrying other OXA-type genes (n = 25). Using this assay, the *bla*OXA-23 gene was detected in all 15 carbapenem-resistant strains of *A. baumannii* collected between 1995-2017. The novel assay proved to be a rapid, robust and inexpensive approach to detection of the commonest putative gene of carbapenem-resistance in *Acinetobacter* spp.

Keywords: bla*OXA-23 gene,* Acinetobacter*, PCR, SYBR Green, carbapenem-resistance*

Introduction

Clinical infections with carbapenem-resistant *Acinetobacter* spp. are refractory to empiric therapy. Due to high mortality and association with outbreaks, they are cause for international concern (Peleg *et al* 2008). The *bla*OXA-23 gene is the most common cause of carbapenem-resistance in clinical isolates of *Acinetobacter* spp. (Peleg *et al* 2008). It may be located on the bacterial chromosome or plasmid, and is thus transferable between strains by molecular mechanisms including integrons (Dolapçi *et al* 2010; Lupo *et al* 2017).

Identifying the mechanism of resistance is an essential step in determining the presence of an outbreak. Weak *in vitro* hydrolysis of carbapenem antibiotics by OXA-encoding enzymes limits phenotypic detection. For this reason, the Rapid Carba-NP test (bioMérieux, USA) which is used to detect carbapenemase activity in bacterial suspensions has poor sensitivity for detection of these enzymes (van der Zwaluw *et al* 2015). False negative results have also been reported with the carbapenem inactivation method (CIM) on testing isolates that harbor an OXA enzyme (Barraud *et al* 2010). Thus, a high index of suspicion is required in order for these isolates to be referred for further testing.

Confirmation of a suspected OXA-carrying isolate is dependent on PCR-based methods for which there are no commercially available assays. The development and implementation of these methods are expensive, limiting widespread application in routine laboratories. To address this we sought to develop an inexpensive, rapid and robust molecular method of identifying the $bla_{\text{OXA-23-like}}$ gene in a collection of clinical isolates of *Acinetobacter* spp. This work followed a previously developed, highly specific, real time SYBR Green-based PCR method that utilizes 75% less reagents than the standard reaction volume of 20.0 µL (McIver *et al* 2017). Specificity is further enhanced by a melt curve analysis of the PCR product and the number of cycles required for its amplification (crossing-point) (McIver *et al* 2017).

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Materials and methods

Bacterial strains

A collection of 25 OXA-containing strains including 11 strains of *Acinetobacter baumanii* with OXA-23 from a reference laboratory were used to develop the assay. An additional 12 type culture strains and 63 clinical strains were used to assess specificity (Table 1). All strains were inoculated on horse blood agar and incubated overnight in air at 35-37°C before testing. Their identity was confirmed using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Germany). Susceptibility to carbapenem antibiotics was tested by disc diffusion using the Calibrated Dichotomous Susceptibility (CDS) method (Bell *et al* 2018). Additionally, the presence of class B metallo-β-lactamase as the cause of carbapenem-resistance was excluded by testing for inhibition of carbapenemase activity using the EDTA chelation method (Bell *et al* 2018).

Novel real-time PCR assay

The PCR assay was performed on growth from an overnight culture suspended in 2.5 mL of sterile water (Braun, Germany) and vortexed for 30 secs to achieve a final suspension approximating a 0.5 McFarland Standard (McFarland Equivalence Turbidity Standard, Remel™). A volume of 3.6 µL of this suspension (above) was added to 1.4 µL of reaction mixture which included 1.0 µL of LightCycler® FastStart DNA Master PLUS SYBR Green 1 (Roche Diagnostics, Germany), and 0.2 µM each forward and reverse primers. The primers amplify a 107 base pair segment of the $bla_{\text{OXA-23-like}}$ gene as described by Yang and Rui (2016). The specificity of the primers was confirmed by a BLAST search (26.09.2019) (http://www.ncbi.nlm.nih.gov/).

Thermocycling was performed using a LightCycler 2.0 (Roche Diagnostics) as programed: 95°C for 10 min to extract bacterial DNA and activate DNA polymerase; 50 amplification cycles at 95°C for 10 secs, 50°C for 10 secs, and 72°C for 20 secs with single acquisition in Channel F1 (530 nm); melt curve analysis commencing at 95°C, reducing to 65°C for 1 min and returning to 95°C in increments of 0.1°C/sec with continuous acquisition; and cooling to 40°C for 30 secs. The melting temperature of the PCR product was calculated as the first derivative of the maximum change in absorbance across the temperature range (as programed). Isolates shown to carry a $bla_{\text{OXA-23-like}}$ gene were also evaluated for the presence of a class 1 integron using a real-time PCR assay as previously described (White *et al* 2001). Statistical analysis was performed using PRISM version 5 (GraphPad®, USA).

Results

The 11 isolates of *A. baumannii* molecularly characterised as carrying the $bla_{\text{OXA-23-like}}$ gene had a crossing-point range of 5-16 cycles (i.e. number cycles for detectable amplification to occur) and a melting temperature mean of 77.3°C (95% CI = 77.0 – 77.5°C). These findings were compared to that of 22 isolates including a carbapenem-sensitive strain of *A. baumannii* and carbapenem-resistant strains of the same species harbouring OXA-24 (n = 3), OXA-48-like (n = 1), and OXA-58 (n = 3) genes; E*scherichia coli* OXA-73 (n = 1); *Klebsiella pneumoniae* OXA-73 (n = 1); and a diverse range of strains listed in Table 1 (n = 12). For these strains, the crossing-points ranged 20-37 cycles and with a melting temperature outside of the range recorded for OXA-23-like positive strains (Figure 1). Two strains with melting temperatures of 76.2°C and 81.0°C were discernible from the OXA-23 positive strains by their crossing-point values. The mean of the crossing-points for OXA-23-positive and -negative strains was statistically different at the 5% level ($p < 0.001$). Thus, the detection of OXA-23 strains is predicated on the rapid amplification of a product with a characteristic melting temperature as shown in Figure 1.

The assay was used to screen for the presence of the $bla_{\text{OXA-23-like}}$ gene in 75 bacterial strains previously tested for carbapenem susceptibility including: collection type cultures (n = 12); unspeciated *Acinetobacter* strains isolated between 1995-2017 (n = 34); and recent clinical isolates of Gram-negative bacilli (n = 29). Here, the gene was detected in all *A. baumannii* isolates (n = 15) with phenotypic resistance to carbapenem (Table 1).

All OXA-23-like-positive *Acinetobacter* spp. were resistant to other classes of ß lactams including aztreonam, third and fourth generation cephalosporins and ampicillin. One OXA-58-like carrying strain of *A. baumanii* and two OXA-48 carrying strains of *E. coli* had a phenotypic borderline annular radius of 6mm with colonies occurring within the zone of inhibition, and were resistant to other ß-lactams. All other OXA-carrying strains tested were resistant to carbapenems.

All 26 strains OXA-23 carrying strains were tested for class 1 integron which is a common mechanism assisting horizontal transfer of resistance genes in Gram-negative bacilli. Here, class 1 integrons were detected in 11/26 (42.3%) of the strains shown to carry a $bla_{\text{OXA-23-like}}$ gene. All isolates with a class 1 integron were resistant to sulphafurazole, whilst 6/15 (40%) of strains without a class 1 integron remained susceptible to sulphafurazole.

Table 1. *Testing of 100* Acinetobacter *spp. and unrelated bacterial strains for* $bla_{\text{OXA-23-like}}$ *gene using in-house PCR assay.*

Strains	*Known bla*-gene	*n*	PCR positive *bla*-OXA-23	Carbapenem resistant
***bla*-OXA-positive strains used to assess specificity**				
Acinetobacter baumanii	OXA-23-like	10	10	10
Acinetobacter baumanii	OXA-23-like, OXA-24	1	1	1
Acinetobacter baumanii	OXA-24	3	0	3
Acinetobacter baumanii	OXA-48-like	1	0	1
Acinetobacter baumanii	OXA-58-like	3	0	3
Escherichia coli	OXA-48	2	0	2
Escherichia coli	OXA-48, NDM	1	0	1
Escherichia coli	OXA-73	1	0	1
Klebsiella pneumoniae	OXA-48	2	0	2
Klebsiella pneumoniae	OXA-73	1	0	1
Type strains*		12	0	0
***Acinetobacter* spp. isolated between: 1995-2017**				
Acinetobacter baumannii	-	15	15	15
Acinetobacter spp.	-	19	0	0
Recent clinical isolates				
Acinetobacter baumannii	-	1	0	0
Enterobacteriales†	-	27	0	1‡
Pseudomonas aeruginosa	-	1	0	0

* Type strains: Carbapenem-sensitive: *Acinetobacter baumanii* (ATCC 19606), *Escherichia coli* (ACM 5185, ACM 5186), and *Pseudomonas aeruginosa* (ACM 5189). Not known to carry *bla*-OXA genes: *Enterococcus faecalis* (ACM 5184), *Enterococcus faecium vanB* (St George Hospital wild strain), *Haemophilus influenzae* (ACM 5187, ACM 5188), *Staphylococcus aureus* (ACM 5190), *Streptococcus agalactiae* (ATCC 12386), *Streptococcus pyogenes* (ACM 5203) and *Streptococcus pneumoniae* (ACM 5191).

† Recent clinical isolates – *Enterobacteriales*: *Escherichia coli* (n = 17); *Proteus mirabilis* (n = 2); *Klebsiella* spp. (n = 5); *Enterobacter* spp. (n = 2); *Serratia marscens* (n = 1).

‡ Mechanism of resistance was not elucidated in this study.

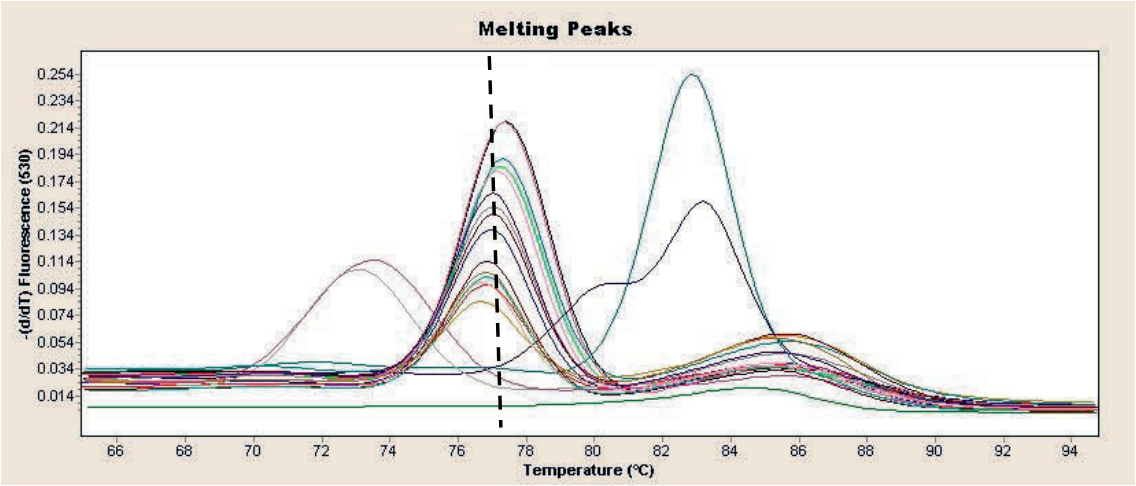

Figure 1. *Melting curves generated by Acinetobacter baumanii with a $bla_{OXA\text{-}23\text{-}like}$ positive gene [the dotted line represents the average melting curve of 77.3°C (95% CI = 77.0 – 77.5°C)] compared to other bacterial species used to assess specificity.*

Discussion

We have developed a rapid, robust and inexpensive PCR assay that detected the $bla_{OXA\text{-}23\text{-}like}$ gene in laboratory isolates of *Acinetobacter* spp. The SYBR Green intercalating dye as a fluorescence marker of amplification provided a cheaper, quicker approach than conventional real-time PCR assays using hybridization probes. As described in the development of a similar assay (McIver *et al* 2017) circumvention of an extraction method in sample preparation (i.e. time saving and cost) and minimizing quantities of reagents with a reduction from the standard reaction volume of 20.0 µL to one of 5.0 µL lowered the cost by 75% per test. Furthermore, this novel assay was also rapid to perform and shown to be highly specific.

A heavy bacterial suspension resulted in a large amount of available DNA. While this increases the test sensitivity, there is increased interference due to non-specific binding to DNA that has the potential to compromise specificity. The broad range of crossing-points that results from non-specific intercalation of SYBR Green dye with DNA has been noted previously, and is the reason for using melting temperatures as the supplementary discriminating tool. Whilst OXA-73 is included in the OXA-23-like group (Evans and Amyes 2014), a different melting temperature was shown (Figure 1). The BLAST search for matches to the primer sequence used in this PCR assay included those genes described within the OXA-23-like group, encompassing but not limited to OXA-23 and OXA-73 (Evans and Amyes 2014).

Phenotypic susceptibility testing supported the observation that a high index of suspicion is required in order to alert the pathologist to the possibility of an OXA-carrying strain. All but one isolate had either a sensitivity zone size or shape indicating non-susceptibility to carbapenems and all isolates were resistant to multiple different ß-lactams to other ß-lactams. Thus, further evaluation is warranted not only on isolates with overt phenotypic resistance to carbapenems but should also be considered for isolates with resistance to other ß-lactams. Unlike a previous report where class 1 integrons were noted in 93% of 89 carbapenem-resistant *A. baumanii* strains (Dolapçi *et al* 2010) in our setting a class 1 integron was implicated in only 42% of OXA-23-like carrying strains of *A. baumanii.*

One of the strengths of this study is the inclusion of a variety of OXA- carrying strains that were obtained to assist in determining specificity between groups. The sequence similarity of the OXA family mandates exclusion of assay cross-reactivity (Evans and Amyes 2014). Limitations of this study include the low prevalence setting which impeded clinical assessment as no positive isolates were identified in the study period. Prospective data collection with confirmation of $bla_{OXA\text{-}23\text{-}like}$ gene carriage is ongoing. The dominance of $bla_{OXA\text{-}23\text{-}like}$ genes in antecedent carbapenem-resistant *A. baumanii* strains is consistent with the literature (Peleg *et al* 2008; Lupo *et al* 2017; Gagnaire *et al* 2017). The isolation of these strains was sporadic, and whilst there was no evidence of space-time clustering to suggest an unidentified outbreak in our hospital, the collection of isolates was not all-inclusive. This assay will allow more rapid identification of $bla_{OXA\text{-}23\text{-}like}$ genes with minimal additional usage of laboratory resources, and is now incorporated into the laboratory workflow.

Acnowledgements

The authors are grateful for the support of colleagues in the Microbiology Department (NSW HP), St George Hospital, Kogarah.

Conflict of interest

There is no conflict of interest for this study.

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ORIGINAL ARTICLE

In search of urinary biomarkers in endometriosis

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Abstract

It is possible to use the rapidly advancing proteomic technologies to identify a protein biomarker or a panel of urinary protein biomarkers for the diagnosis of endometriosis. The identified proteins may play potential biological roles in endometriosis and thus may contribute towards the elucidation of the pathogenesis of the disease. Additionally, some of these proteins may have relevant influences with endometriosis-associated infertility.

Many biological specimens, such as peritoneal fluid, serum, urine, endometrial tissue and menstrual fluid, have been used in an attempt to find a biomarker or a panel of biomarkers suitable for the diagnosis of endometriosis. To date, the success of such an undertaking is still some distance away. The development of a sensitive and specific screening test for endometriosis is a priority and employment of proteomic technologies may present us with an answer. A successful development of a screening test for the diagnosis of endometriosis will have a huge social and economic impact worldwide and additionally establish the true prevalence of the disease.

Keywords: endometriosis, biomarker, urine, diagnosis, proteomics

Introduction

Endometriosis is a benign, oestrogen-dependent gynaecological disorder defined by the growth of endometrial-like tissue outside the uterus (Fourquet *et al* 2010; Lessey 2000; McLeod and Retzloff 2010). This endometrial-like tissue is made up of both stroma and glands from endometriotic lesions (Maharajaa *et al* 2019). These are frequently detected in multiple pelvic and visceral surfaces including the pelvic peritoneum, ovaries, pouch of Douglas, rectovaginal septum, rectum, bladder and ureter (Koninckx *et al* 1991). Rarer are endometriotic lesions in locations distant from the pelvic cavity, including the umbilicus, appendix, lung, pleura, pericardium and brain (Fraser 2008; Wang *et al* 2009).

The prevalence of endometriosis is 5-15% of women in their reproductive years (Berbic and Fraser 2011; Fassbender *et al* 2013; Giudice and Kao 2004; Heinig *et al* 2002; Nasu *et al* 2009) and rising up to 30-50% in women with infertility and/or pain (McLeod and Retzloff 2010; Meuleman *et al* 2009; Rogers *et al* 2009). Although a majority of affected women are of reproductive age, endometriosis has also been documented in pre-menarchal girls and postmenopausal women (Overton *et al* 2007) and in adolescents (Nguyen *et al* 2018). The economic impact of endometriosis is compounded by the latency in the diagnosis, especially in young women that delay seeking medical treatment. Owing to the common misinterpretation of endometriosis-induced pain as being menstrual-related abdominal pain, the diagnosis of endometriosis is typically delayed by 8-10 years.

Adolescent girls who suffer from the symptoms of endometriosis delay seeking medical attention by 4.6 years, and by the time they seek medical attention, it typically takes another 4.7 years until diagnosis (Greene *et al* 2009). Ballweg (2004) reported an increase of endometriosis-like symptoms in girls before the age of 15 years as well as an earlier onset of menarche, indicating the potential need to screen adolescent and younger girls as early as they display symptoms of endometriosis for confirmatory diagnosis.

Endometriosis is a highly variable disease in terms of the presenting symptoms, anatomical severity, rate of progression, response to treatment and rate of reoccurrence

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(Fraser 2008). The symptoms can be non-specific (May *et al* 2010), however, often include various types of pelvic pain, premenstrual spotting, heavy menstrual bleeding and infertility (Fraser 2008).

Pain

Most women with endometriosis describe some form of pelvic pain (Sinaii *et al* 2008), affecting around one in five women (Bush *et al* 2011). This pain is often debilitating and has a negative impact on their ability to work, personal relationships and self-esteem (Huntington and Gilmour 2005; Mathias *et al* 1996). The burden of painful endometriosis has been characterised by the limitations that affect all areas of their daily life (Fourquet *et al* 2010).

Women diagnosed with endometriosis can exhibit a range of symptomatic abdominal pelvic pain symptoms (Markham *et al* 2019) whilst some may experience asymptomatic pelvic pain symptoms (Markham *et al* 2020). The predominant pelvic pain symptom is period pain (dysmenorrhoea), which is often rated as severe. Other pain symptoms can include painful bowel motions (dyschezia), pain with urination (dysuria), painful intercourse (dyspareunia), pain radiating down the legs, painful abdominal bloating and discomfort and chronic pelvic pain (CPP) (Fraser 2010; Luscombe *et al* 2009). This cluster of pain symptoms may be thought of as endometriosis-associated pain (EAP) (Morotti *et al* 2014).

Chronic, or persistent pelvic pain is regarded as prolonged pain when experienced for greater than six months. Chronic recurrent pain incorporates pain-free episodes and extends over months or years (Gatchel *et al* 2007). In women with endometriosis, CPP which incorporates chronic recurrent pain is unrelated to menstruation or ovulation (Ballard *et al* 2010). Most women with endometriosis retrospectively report that persistent pelvic pain symptoms began in their adolescent years.

Endometriosis is a highly variable condition, yet many women with significant endometriosis will experience some form of pelvic pain (Fraser 2008). The variability of the pelvic pain and the lack of direct association with the disease phenotypes is suggestive that the disease and neural contributors to chronic endometriosis-associated pain are more complex than previously thought.

Infertility

The fecundity rate in normal reproductive-age couples with unexplained infertility is estimated to be around 15-20%, whereas fecundity rate in women with untreated endometriosis is estimated to range from 2-10%. Women with mild endometriosis have been shown to have a significantly lower probability of pregnancy during a period of three years than do women with unexplained infertility (36 vs 55%, respectively) (Macer and Taylor 2012).

Heritability

There is evidence that endometriosis is a heritable disease. Matalliotakis (2008) reported an 11.6-fold increase in the risk of endometriosis among sisters and an 8-fold increase among mothers of those diagnosed with endometriosis. There is also a high concordance of endometriosis among identical twins (Hadfield *et al* 1997; Moen 1994). Recent genome-wide association studies have identified a number of chromosomal loci for endometriosis risk and have shown that the genetic burden correlates with anatomical disease severity (Nyholt *et al* 2012; Rahmioglu *et al* 2014; Sapkota *et al* 2015). A strong familial linkage has also been reported in non-human primates (Zondervan *et al* 2004) and this has lent support to a genetic predisposition to the disorder.

Cost

It is estimated that more than 700,000 Australian women, girls and other individuals are living with endometriosis. Endometriosis is reported to cost more than $7.7 billion dollars in healthcare, absenteeism and lost social and economic participation (Tariverdian *et al* 2007).

Diagnosis

The gold standard for the diagnosis of endometriosis is laparoscopy accompanied with histological confirmation (Dunselman *et al* 2014). However laparoscopy has some limitations in terms of being invasive, costly and carries the possibility and implications of false negative findings. These include mistaking lesions for corpus luteum cysts and missing a peritoneal or deep lesion in difficult locations. Other issues include the potential inaccuracy of confirmation of diagnosis by histopathology of biopsied lesions, as sometimes the lesions are not included in biopsies taken. Additionally, inexperienced histopathologists can fail to identify the characteristic features of endometriosis, especially in cases of mild disease, where glandular elements may not be obvious (Al-Jefout *et al* 2009).

Since 2006 clinicians D'Hooghe *et al* (2006) and D'Hooghe and Hummelshoj (2006) have been urgently calling for a non-invasive diagnostic test for endometriosis to prevent the long delays experienced between the initial onset of symptoms and the diagnosis. There is still a long delay, from the onset of symptoms until a definitive diagnosis is made, which can take eight to ten years (Ahn *et al* 2017).

The consensus of the World Endometriosis Society has been that the development of a reliable non-invasive diagnostic test is one of the top research priorities in endometriosis (Rogers *et al* 2017; Rogers *et al* 2013). In 2014, the World Endometriosis Research Foundation had advised and guided researchers to standardise methods of sample collection and analysis of data (Becker *et al* 2014; Fassbender *et al* 2014). The Australian Government's

National Action Plan for Endometriosis (2007) has emerged from the combined efforts of patients, advocacy groups, clinicians, researchers and parliamentarians to have endometriosis acknowledged as a chronic condition and a substantial health burden in Australia. This has included the funding and commissioning of research into the development of a non-invasive diagnostic test for endometriosis.

Importantly, a delayed diagnosis of endometriosis increases the negative impacts on women's lives, including their psychological, social and relationship wellbeing (Culley *et al* 2013; Fourquet *et al* 2011; Gao *et al* 2006; Gilmour *et al* 2008). To date the hunt for a non-invasive biomarker or a panel of biomarkers for endometriosis remains an ongoing and challenging issue for early diagnostic testing.

Urine as a possible biomarker

Human urine is a biological fluid which contains a wealth of biological biomarkers and is readily obtainable. It contains up to 150mg/24h of excreted proteins (Gonzalez-Buitrago *et al* 2007a). Normal urinary proteins can originate from glomerular filtration of plasma proteins. Other sources of urinary proteins could include proteins secreted from renal tubular epithelial cells, shedding of whole cells along the urinary passage and of the membranes of the renal tubular epithelial cells and also from exosome secretion (Hoorn *et al* 2005; Pisitkun *et al* 2006). Low molecular weight proteins, less than 40kDa, readily pass through the glomerular filtration barrier and are reabsorbed by the proximal renal tubules. When the glomerular filtration barrier and the tubular reabsorption mechanism are compromised by disease, an increased amount of plasma proteins are allowed to pass into the ultrafiltrate and subsequently the urine (Gonzalez-Buitrago *et al* 2007a).

In a previous study, gel-based proteomic analyses have been successfully used in the search for urinary biomarkers for various glomerular diseases (Thongboonkerd *et al* 2004). The secretion and excretion of proteins by the urinary tract vary from non-diseased to diseased state/situation. Potentially, the urinary proteome could be different between a control patient and one with endometriosis and thus urine could provide a possible biomarker for the early non-invasive diagnostic screening measure for endometriosis.

Urine collection and storage

The first morning urine sample is thought to be a suitable one (Thongboonkerd 2007). However, potential bacterial contamination may occur due to a long residence time in the bladder (Russo *et al* 2002). No significant changes in the urine proteome were observed in urine samples stored at -20°C for several years, however, urine samples were found to be more stable when stored at -70°C (Dakna *et al* 2009; Klasen *et al* 1999; Weissinger *et al* 2007). Zerefos and Vlahou (2008) reported occasional changes of protein profile were observed for a 24-hour storage at 4°C, thus a shorter storage time, up to 6 hours at 4°C, would be a better choice (Wu *et al* 2010). A male or female adult human excretes 30-130mg of protein and 22mg of peptides daily (Brenner and Rector 2004; Strong *et al* 2005).

Results obtained by Lafitte (2002) yielded more proteins present in the morning when compared with pooled 24-hour urine. The highest number of protein spots were discovered in morning urine collections and the proteome pattern was found to have differed significantly (Khan and Packer 2006). Twenty-four-hour urine collections are time consuming and inconvenient for all patients and are subject to collection errors. The protein-creatinine ratio, which is measured with a single spot urine sample, corrects for variation in urinary protein concentration due to hydration. There have been reports of good correlations between the protein-creatinine ratio and results obtained from a 24-hour urine collection (Chitalia *et al* 2001; Eslamian *et al* 2011; Schwab *et al* 1987; Steinhauslin and Wauters 1995).

Advantages of using urine

The use of urine is superior to blood in searching for a non-invasive biomarker or panel of biomarkers. Urine is considered as the most clinically useful biological fluid used for proteomic investigation due to several advantages such as, its collection is non-invasive, easy to obtain in large amounts, and enables easy repeat sampling if desired (Wu *et al* 2010). Additionally, the proteins and peptides are quite stable and remain in solution. In contrast blood collection is invasive and requires meticulous pre-analytical handling and its proteomic analysis is prone to analytical artefacts (Fliser *et al* 2007). A detailed comparison of serum and plasma proteomes has revealed that an array of proteases are activated immediately upon clotting, resulting in the generation of many degradation products (Kolch *et al* 2005). Fliser *et al* (2007) reported that proteins in biological fluids may degrade rapidly when handled inappropriately, however, urinary proteins have been shown to remain stable long enough to perform reliable proteome analysis.

Limitations of using urine

Human urine does have its limitations. Many can be overcome through additional steps in experimental protocol. Human urine has a diluted protein concentration with a high-salt content, which interferes with proteomic analysis through an adverse role in the isoelectric focusing of two-dimensional gel electrophoresis (2DE). Additional preparative steps are required to concentrate urinary proteins and reduce the presence of high salt content. A dilute urine protein concentration can be further concentrated up to 1000-fold using ultrafiltration steps

with several washes (Lasne and de Ceaurriz 2000; Lasne et al 2002). Samples have a wide variability in protein concentrations but this could be compensated by a standardisation based on urinary creatinine (Vestergaard and Leverett 1958). Circadian fluctuations in the urinary proteome could arise due to diet, exercise, metabolic and catabolic processes. These could complicate defining disease specific markers (Fliser *et al* 2007). As a consequence, the reproducibility of the assay has been found to have been reduced and thus highlight the importance of a standardised protocol for urine sample collection and preparation (Gonzalez-Buitrago *et al* 2007b). When using urine in proteomic research, it is recommended to include (1) a clear clinical objective (2) protocols for sampling and sample preparation (3) addition of protease inhibitors, (4) sample storage and effects of freeze-thaw cycle, (5) sample preparation methods for concentrating urinary proteins, (6) choice of proteomic platforms with high throughput and high reproducibility (7) use of appropriate statistical methods with a clear clinical question (8) validation of the potential objectives by analysing larger subject groups and finally (9) sequencing of potential biomarkers (Thongboonkerd 2007; Wu *et al* 2010).

Challenges of proteomic research

Proteomic research has played a central role in the discovery of disease biomarkers and drug targets. Mass spectrometry (MS) used in concert with many separation methods is the principal methodology for proteomics (Aebersold and Mann 2003). Two fundamental strategies for protein identification and characterisation by MS currently are employed in proteomics. These are peptide-based bottom-up (Gregorich *et al* 2014) and protein-based top-down approaches (Chait 2006; Gregorich *et al* 2014; Wang *et al* 2014). Inconsistencies between studies can arise due to there being several available proteomic platforms, and also through varying sensitivities and molecular weight ranges. Therefore detailed knowledge of these platforms is essential for the accurate interpretation of results. A proteome from any tissue or a biological fluid has an extreme complexity and a dynamic range of protein candidates (Anderson and Anderson 2002). Anderson (2005) reported that there are no clearly preferred platforms for the discovery of biomarkers and certainly no comprehensive platforms.

Modifications and identifications from recovered peptides may be achieved, however the modification status of the unrecovered sequence portion remains unknown (Zhang and Ge 2011). There is a loss of connectivity between post translation modification (PTM) resulting from the peptide loss during sample preparation and sample digestion (Gregorich *et al* 2014). Furthermore, as each protein is digested into many small peptide components, the overall complexity of the sample is increased (Zhang and Ge 2011). Researchers continue to face challenges when screening for biomarkers in which the protein candidates are below 30kDa. Smaller proteins and peptides have fewer cleavage sites and often do not generate enough peptides for confident identification (Han *et al* 2008).

Proteomic platforms

Proteomic platforms are classified as either top-down or bottom-up (Cui *et al* 2011; Tipton *et al* 2011). Top-down proteomics analyse the naturally occurring intact proteins. The qualitative and quantitative differences of the proteins present in different samples can be assisted in disease biomarker characterisation. Bottom-up platform analyses the mixture of proteolytic fragments of a given protein, connecting each detected fragment to the parent protein.

Bottom-up proteomics

The classical bottom-up strategy is a typically peptide-based MS proteomics. Also known as shotgun proteomics, it involves in-gel or in-solution proteolytic digestion of proteins with enzymes, usually trypsin into many pieces of smaller peptides before MS analysis (Bogdanov and Smith 2005; Chait 2006; Han *et al* 2008; Yates *et al* 2009). This approach is well suited for protein identification from the database (Steen and Mann, 2004). Using the bottom-up strategy, methods for relative and absolute quantitation of protein expression are well developed (Gregorich *et al* 2014). Bottom-up proteomics serves as a workhorse in modern proteomics, with high throughput and automation (Zhang and Ge 2011). The bottom-up approach has intrinsic limitations in characterising protein modifications as only a small and variable fraction of peptides are recovered from digestion.

Top-down proteomics

Top-down proteomics, in contrast with the bottom-up strategy, analyse intact proteins without proteolytic digestion, avoiding as much as possible any sample alteration (Tipton *et al* 2011). Traditionally, protein-based top-down studies have primarily focused on the analysis of a single protein or small number of proteins typically obtained via affinity purification (Ayaz-Guner *et al* 2009, Zabrouskov *et al* 2008). This approach faces less sample complexity and preserves all information related to the status of the intact protein, including PTMs and sequence variations arising from mutations, truncations and alternative splicing events (Siuti and Kelleher 2007; Zhang and Ge 2011). It is a powerful technology for the comprehensive analysis of protein modifications (Ge *et al* 2002; Ge *et al* 2009; Han *et al* 2006; Siuti and Kelleher 2007; Zabrouskov *et al* 2008). This strategy preserves the labile structural characteristics that are mostly destroyed in bottom-up proteomics (Siuti and Kelleher 2007). Unfortunately, the physical and chemical diversity of intact

proteins are much greater than that of peptides. The large-scale separation of intact proteins is challenging.

Top-down proteomics has developed through a variety of stages, with numerical technical challenges in sample preparation and has required improvement in instrumental sensitivity detection limit throughout and automation. The bottom-up proteomics is better established. (Armirotti and Damonte 2010; Garcia 2010; Kelleher 2004; Kellie *et al* 2010; Siuti and Kelleher 2007). The incorporation of the novel tandem mass spectrometry (MS/MS) technique has greatly enhanced the capability of the top-down MS in the structural analysis of biomolecules. In many respects, bottom-up and top-down approaches are complementary in proteomics.

2-dimensional electrophoresis (2DE)

A common concern with 2-dimensional electrophoresis (2DE) is the long experimental protocol of sample preparation, separation, visualisation and data analysis; small errors compound which challenge meaningful quantitation (Freeman and Hemby 2004). However research is continuing to face challenges when screening for biomarkers in which the protein candidates are below 30kDa. Smaller proteins and peptides have fewer cleavage sites and often do not generate enough peptides for confident identification (Han *et al* 2008).

Trypsin digestion

The most important step in sample preparation for shotgun proteomics is the conversion of proteins to peptides and this key step is protein digestion (Hustoft *et al* 2011). Trypsin is the most commonly used protease for this purpose since it has a defined specificity. It hydrolyses only the peptide bonds in which the carbonyl group is followed by an arginine or lysine residue, with the exception of lysine and arginine which are N-linked to aspartic acid. Similarly, the cleavage does not occur if proline is positioned on the carboxyl side of lysine and arginine (Hustoft *et al* 2012; Rodriguez *et al* 2008). Trypsin has poor thermal stability and must be stored at very low temperatures (-20^{o}C to -80^{o}C) to prevent autolysis. It performs best as an in-solution tryptic digestion at a temperature of 37^{o}C (Havlis *et al* 2003), however, its usage in elevated temperatures has been suggested (Capelo *et al* 2009).

Post-translational modifications

Proteins are synthesised by ribosomes translating mRNA into polypeptide chains. The synthesised polypeptide chains may then undergo PTM with the addition of functional groups covalently-bound on the amino acid side chains or at the protein's C- or N- termini to form the mature protein product (Pratt *et al* 2006). The most common PTM of protein is phosphorylation, that is, the addition of a phosphate group to an amino acid. It is one of the central reversible PTMs that regulate cellular metabolism, protein-protein interaction, enzyme reactions, protein degradation for a myriad of proteins which results in intercellular signalling cascades (Ghosh and Adams 2011; Hunter 1995). Phosphorylation affects the physiological and structural implication, activation or deactivation of the proteins and it increases the molecular weight of the protein. Other common PTMs of proteins are, glycosylation, acetylation and proteolysis. These covalent modifications of a protein can modulate function of a protein (Mann and Jensen 2003). Aberrant protein PTMs together with mutations and alternatively spliced isoforms are increasingly recognised as important underlying mechanisms for many diseases (Biesiadecki *et al* 2002; Jin *et al* 2008; Yuan and Solaro 2008).

What is a biomarker?

A biomarker is a characteristic objectively measured and an indicator of a biological process (Nonaka *et al* 2012). Biomarker is a term often used to refer to a protein measured in blood whose concentration reflects the severity or presence of some disease state. However protein biomarkers are associated with a huge range of possible PTM (Schrattenholz and Groebe 2007). There is a crucial demand for improved resolution in separation technologies. The ultimate test is the subsequent clinical validation, testing the biomarker in clinical settings and using large numbers of patient samples. Ultimately a successful screening program must be cost effective and non-invasive (Pavlou *et al* 2013). Molecular biomarkers provide a dynamic and powerful approach to understanding the spectrum of diseases with applications to epidemiology, prevention, diagnosis, treatment and management (Galasko 2001; Reiber and Peter 2001). Proteomic technologies have produced potential biomarkers in many areas of health and have played significant roles in diagnosis and in drug development (Chang 2007; Slamon *et al* 2001).

However, the journey of a protein biomarker from the bench to the clinic is long and challenging. Many potential biomarkers have failed to reach the clinic as they have fallen short in their ability to contribute decisively to patient care, except for providing some incremental, nevertheless, clinically non-essential information (Kantelhardt *et al* 2011).

Urinary biomarkers in endometriosis

An accurate diagnosis of endometriosis requires an invasive surgical procedure, therefore the development of a non-invasive diagnostic test using the urinary proteome, could have substantial benefits. A diagnostic urinary biomarker or a panel of biomarkers may indicate which women require a laparoscopy and thus eliminating unnecessary surgical investigations (May *et al* 2010). Large well-designed studies together with increased knowledge of the pathogenesis of

endometriosis will improve overall health-related quality of life for patients suffering from this debilitating disease.

The invasiveness of surgery for the diagnosis of endometriosis has been a major obstacle in the early diagnosis (Yun *et al* 2014). Also, it is unlikely that women of reproductive age would subject themselves to such an invasive surgery when they could opt to temporarily diminish their symptoms by means of other therapeutic mechanisms (Ahn *et al* 2017).

Fassbender (2015) described the status of biomarkers of endometriosis in urine, and noted that since 2010, only 11% of reported endometriosis biomarker studies were based on investigations using urine (Rizner 2014). Liu (2015) provided a Cochrane systematic review on urinary biomarkers for the non-invasive diagnosis of endometriosis. It was concluded that there was insufficient evidence for the recommendations for any urinary biomarker for use as a replacement or triage test in clinical practice for the diagnosis of endometriosis.

Research into urinary biomarkers for endometriosis has been continuous (Gueye *et al* 2017). The last couple of decades has seen numerous publications emerging: (1) Urine vascular endometrial growth factor-A is not a useful marker for endometriosis (Potlog-Nahari *et al* 2004); (2) Evaluation of serum and urinary angiogenic factors in patients with endometriosis (Cho *et al* 2007); (3) Matrix metalloproteinases are elevated in the urine of patients with endometriosis (Becker *et al* 2010); (4) Discovery of a novel biomarker in the urine in women with endometriosis (Tokushige *et al* 2011); (5) Urinary peptide profiling identifies a panel of putative biomarkers for diagnosing and staging endometriosis (El-Kasti *et al* 2011); (6) Urinary vitamin D-binding protein is elevated in patients with endometriosis (Cho *et al* 2012); (7) Evaluation of elevated urinary enolase 1 levels in patients with endometriosis (Yun *et al* 2014); (8) Urine peptide patterns for non-invasive diagnosis of endometriosis: a preliminary prospective study (Wang *et al* 2014); (9) Cytokeratin-19 as a biomarker in urine and in serum for the diagnosis of endometriosis-a prospective study (Kuessel *et al* 2014); (10) CYFRA 21-1 in urine: a diagnostic marker for endometriosis? (Gjavotchanoff 2015); (11) Nuclear magnetic resonance metabolomics profiling of urine provides a non-invasive alternative to the identification of biomarkers associated with endometriosis (Vicente-Munoz *et al* 2015).

Discussion

Endometriosis is a complex gynaecological disease and it has a high prevalence in women of reproductive age. The improvement in the areas of diagnosis, management and therapeutics has been lacking. The identification of novel clinical biomarkers of endometriosis that are sensitive, specific, and predictive for endometriosis is widely recognised as being a high priority (Toor *et al* 2014).

Over the last 25 years, more than 100 possible biomarkers have been investigated as potential diagnostic tests for endometriosis, however none these have proven to be clinically useful (May *et al* 2010). Additionally, May (2010) covered the ups and downs of the search for biomarkers for the diagnosis of endometriosis. Recurrence rates of up to 50% after 5 years of the surgical removal of lesions have been reported (Guo 2009) and it would assist as a disease predictor of recurrence if a biomarker or a panel of biomarkers were available. All these factors contribute to the well-recognised delay in diagnosis of 8-12 years (Hadfield *et al* 1996) and exacerbate the effects on the quality of life of individuals, work productivity and the economic impact of many countries.

Proteomic profiling and utilising Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry (MS) in combination with bioinformatics software for the identification of the actual protein and peptide patterning could offer a distinctive marker/s for a non-invasive diagnosis and offer an earlier treatment management option for endometriosis. However using MALDI-TOF MS does not allow the direct identification of peptides or proteins that are differentially expressed. Similar to serum, urine reflects an amalgam of systemic processes, and it will be important to assess the ability of a urinary assay to differentiate endometriosis from other inflammatory conditions (Burney 2014).

Conclusion

Endometriosis was first described microscopically by Karl von Rokitansky in 1860 (Batt 2011), although its initial recording in medical texts dates back to more than 4,000 years ago (Nezhat *et al* 2012). As can be seen, endometriosis has a long-standing association in human history, however,the challenge of developing a non-invasive biomarker/s for endometriosis whether derived from urine, serum, plasma, peritoneal fluid, saliva or any other biological fluid enters the third decade of the twenty-first century still confined to the research bench.

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Two children present with a pleural effusion and Burkitt cell lymphoma

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Case 1

A three-year-old male child presents with a history of abdominal distension and constipation for three weeks. He had night sweats and wasting of limbs for the same period.

A full blood count is performed with the following results:

Hb	104	RR 107-136 g/L
MCV	74.7	RR 73-85 fL
MCH	23.3	RR 24.8-29.9 pg
WBC	7.96	RR 4.90-12.80 x 10^9/L
Platelet	491	RR 214-483 x 10^9/L

Neutrophil	66.5	%
Lymphocyte	27.0	%
Monocyte	6.4	%
Eosinophil	0.0	%
Basophil	0.1	%
Neutrophil	5.29	RR 1.7-6.7 x 10^9/L
Lymphocyte	2.15	RR 2.0-6.6 x 10^9/L
Monocyte	0.51	RR 0.2-1.0 x 10^9/L
Eosinophil	0.00	RR 0.0-0.6 x 10^9/L
Basophil	0.01	RR 0.0-0.1 x 10^9/L

Red cells are normal for age however the MCH is minimally reduced. White cells and platelets are normal. Iron studies were performed:

IRON	4.9 µmol/L	RR 8.1- 32.6
TRANSFERRIN	2.4 g/L	RR 1.8- 3.5
TRF SAT	8.2 %	RR 20-52
FERRITIN	5 µg/L	RR 20-300

Pleural fluid is received for a cytospin preparation:

CYTOSPIN RESULTS	
WBC count	26.7 x 10^9/L
Total cells counted	100
Burkitt Lymphoma cells	100 %

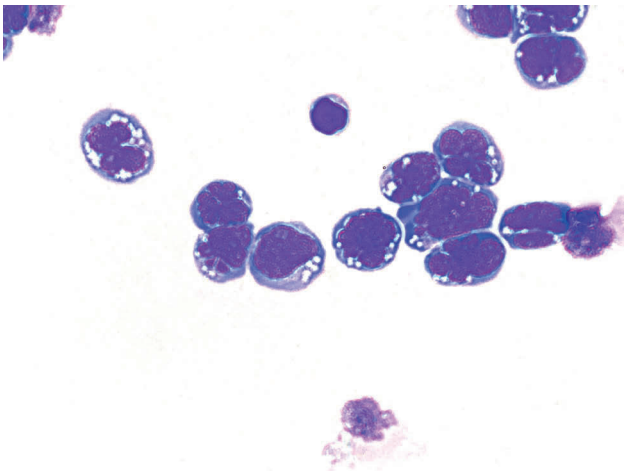


Figure 1. *Burkitt cells in the pleural fluid*

Flow cytometry was performed on the pleural fluid with the following results:

CD45+/HLA-DR+/CD19+/20+/22+/23-/10+/5-/38+/56-/Lambda+

This is a predominantly a B-cell population and the B-cells (approximately 75% of total cells) have the above phenotype. This is a monoclonal B-cell population lambda positive Burkitt cell lymphoma (Figure 1).

Case 2

A seven-year-old male child presents with right chest pain and right pleural effusion. He also has abdominal lymphadenopathy for investigation.

A full blood count is performed with the following results:

Hb	91	RR 110-139 g/L
MCV	70.3	RR 74-86 fL
MCH	23.3	RR 25.5-30.6 pg
WBC	7.84	RR 4.70-12.30 x 10^9/L
Platelet	385	RR 205-457 x 10^9/L

Neutrophil	90.7	%
Lymphocyte	5.5	%
Monocyte	3.8	%
Eosinophil	0.0	%
Basophil	0.0	%
Neutrophil	7.11	RR 1.8-7.7 x 10^9/L
Lymphocyte	0.43	RR 1.6-5.1 x 10^9/L
Monocyte	0.30	RR 0.1-1.0 x 10^9/L
Eosinophil	0.00	RR 0.0-0.6 x 10^9/L
Basophil	0.00	RR 0.0-0.1 x 10^9/L

Although the MCV and MCH are suggestive of a mild iron deficiency, iron studies are not performed on this case. White cells and platelets are normal.

Pleural fluid is received for a cytospin preparation:

CYTOSPIN RESULTS	
WBC	34 x 10^9/L
Burkitt Lymphoma cells	100 %

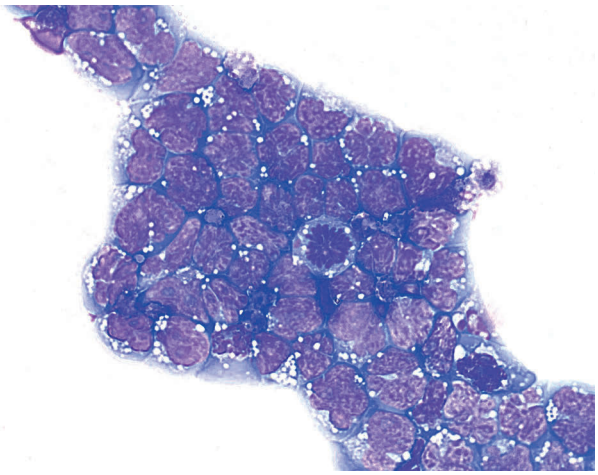


Figure 2. *Burkitt cells in the pleural fluid.*

Flow cytometry is also performed on the pleural fluid with the following results:

CD45+/HLA-DR+/CD19+/10+/22+/23-/200-/38+/5-/FMC7+/Lambda+

Approximately 11% (of the total cells) are B-cells with the above phenotype and this is consistent with Burkitt cell lymphoma.

A bone marrow aspirate is performed on this child.

The cytological features together with immunohistochemistry on the bone marrow confirmed Burkitt lymphoma.

Morphologically the blast cells are large and homogeneous. They have a dense but finely stippled nuclear chromatin pattern; the nucleus is round with one or more prominent nucleoli. The cytoplasm is moderately abundant and intensely basophilic with prominent vacuolation (Figure 2).

Discussion

For two children to present with a pleural effusion within two weeks of each other and to both be diagnosed with a pleural effusion and Burkitt cell lymphoma is very rare.

Burkitt cell lymphoma is a highly aggressive lymphoma and has a very short doubling time.

There are three clinical variants of Burkitt cell lymphoma - Endemic Burkitt cell lymphoma, Sporadic Burkitt lymphoma and Immunodeficiency associated Burkitt lymphoma.

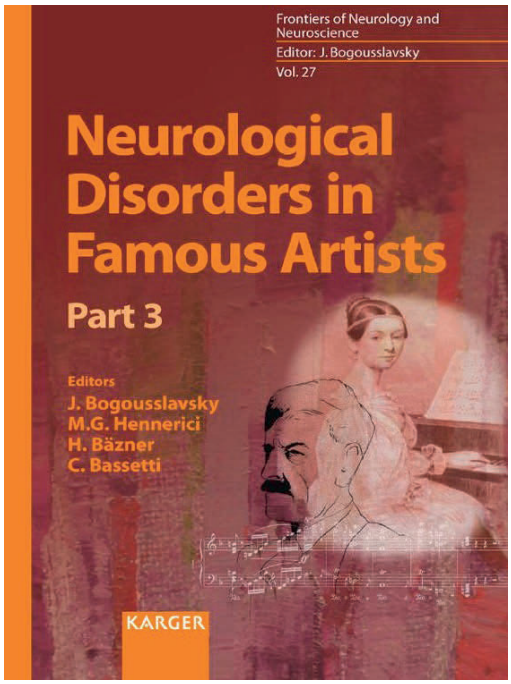
Endemic Burkitt lymphoma occurs in equatorial Africa and Papua New Guinea. It is the most common malignancy of childhood occurring in children between the age of 4 and 7 years.

Sporadic Burkitt lymphoma occurs throughout the world, occurring mainly in children and young adults. It is associated with the Epstein-Barr virus.

Immunodeficiency associated Burkitt lymphoma is primarily associated with the human immunodeficiency virus (HIV).

BOOK REVIEW

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



'Neurological Disorders in Famous Artists' Part 3 is the third book in a four part series. The book is a compilation of seventeen articles that present us with new and unexpected perspectives on neurology and neurological disorders. The first article is about Leonardo da Vinci, arguably the most famous polymath who ever lived. He is analysed as a vegetarian and the question is asked of whether his dietary choices increased his risk of stroke. The authors throughout the book analyse the impact of the artists' diseases on their artwork, music or writing.

German dermatologist and Paul Klee fanatic, Hans Suter, contributes an article on the fatal progression of systemic sclerosis. The article contains fourteen selected photographs of Klee's surrealist and expressionist art including pieces produced with oils, coloured paste, watercolour, pencil, pastels and pen. There is also an interesting graph of Klee's productivity each year during the progression of his disease.

The paper in the hardcover book is semi-gloss and acid-free which is appropriate for the high resolution illustrations of intricate artworks and images of handwritten personal letters. In the article on French literary author, Blasie Cendrars, we can see examples of handwriting in the non-dominant hand after losing a limb in war and his handwritten notes describing the experience of having a phantom limb.

Julien Bogousslavsky is one of the editors, the author of two articles and co-author of one of the articles. He is a Swiss neurologist who has written hundreds of published articles and books on neurology and is deeply passionate about the arts. In his article on Giorgio De Chirico, Bogousslavsky provides his commentary on replay syndrome and painting in series, a common technique used by artists including famous contemporary artists such as Andy Warhol. In an article by mathematician, Ioan James, autism is discussed using the case studies of Andy Warhol and Bela Bartok.

Brandy Matthews, an American neurologist, contributes two articles. Her article "Bravo! Neurology and the Opera" discusses several aspects of opera such as the neuroanatomy of singing, characters in opera who had a neurological disease and the emotional response of opera fans to music. In her article on William Shakepeare's plays, there is a tabulated analysis of the characters who had neurological disorders. The table cites lines from the literature that provide evidence of the signs and symptoms of the disorder.

Other examples of disorders that are addressed are stroke, chronic pain, epilepsy, myalgia, suicidal ideation, post-traumatic stress and migraine. The discussions are anchored with specific and well-referenced examples from the lives of famous artists or characters from literary works. Each article is written in the format of abstract, body, conclusion and reference list.

If you are a medical professional and have an interest in fine arts, music or literature, this is a book worth considering. The book would fall under the genres of biography, history and medical science. This is not an instructional text or an update on the latest treatments and discoveries. But knowledge of the historic development of a disorder can increase understanding. In this book you will undoubtedly find many modern and left field ways to approach science and art.

Melissa Anne Gaudart

Medical Scientist

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Journal-based CPD No. 70
Page 1 of 1

Questions relating to the article *'A rapid, reliable and inexpensive PCR assay for detecting* bla-*OXA-23 in clinical isolates of* Acinetobacter baumannii *using SYBR Green technology'* at page 86 of this issue.

1.	Carbapenem-resistance amongst *Acinetobacter* spp. is commonly associated with the acquisition of the *bla*OXA-23 gene.	True/False
2.	Clinical infections with carbapenem-resistant *Acinetobacter* spp. are refractory to empiric therapy.	True/False
3.	The bla_{OXA-23} gene is the most common cause of carbapenem-resistance in clinical isolates of *Acinetobacte*r spp. (Peleg *et al* 2008).	True/False
4.	The bla_{OXA-23} gene is transferable between strains by molecular mechanisms including integrons (Dolapçi *et al* 2010; Lupo *et al* 2017).	True/False
5.	Weak *in vitro* hydrolysis of carbapenem antibiotics by OXA-encoding enzymes limits phenotypic detection.	True/False
6.	The Rapid Carba-NP test (bioMérieux, USA) has not poor sensitivity for detection of OXA-encoding enzymes (van der Zwaluw *et al* 2015).	True/False
7.	Confirmation of a suspected OXA-carrying isolate isnot dependent on PCR-based methods for which there are no commercially-available assays.	True/False
8.	A collection of 25 OXA-containing strains including 11 strains of *Acinetobacter baumanii* with OXA-23 from a reference laboratory were used to develop the assay.	True/False
9.	All strains were inoculated on horse blood agar and incubated overnight in air at 35-37°C before testing.	True/False
10.	Susceptibility to carbapenem antibiotics was tested by disc diffusion using the Calibrated Dichotomous Susceptibility (CDS) method (Bell *et al* 2018).	True/False

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Journal-based CPD No. 71
Page 1 of 1

Questions relating to the article *'In search of urinary biomarkers in endometriosis'* at page 92 of this issue.

1.	Using the rapidly advancing proteomic technologies to identify a protein biomarker or a panel of urinary protein biomarkers for the diagnosis of endometriosis are possible.	True/False
2.	The identified proteins may play potential biological roles in endometriosis and thus may contribute towards the elucidation of the pathogenesis of the disease.	True/False
3.	Endometriosis is a benign, oestrogen-dependent gynaecological disorder defined by the growth of endometrial-like tissue outside the uterus.	True/False
4.	These are frequently detected in multiple pelvic and visceral surfaces including the pelvic peritoneum, ovaries, pouch of Douglas, rectovaginal septum, rectum, bladder and ureter (Koninckx *et al* 1991).	True/False
5.	Adolescent girls who suffer from the symptoms of endometriosis delay seeking medical attention by 8.6 years.	True/False
6.	Endometriosis is a highly variable disease in terms of the presenting symptoms, anatomical severity, rate of progression, response to treatment and rate of reoccurrence (Fraser 2008).	True/False
7.	Most women with endometriosis describe some form of pelvic pain (Sinaii *et al* 2008), affecting around one in five women (Bush *et al* 2011).	True/False
8.	This cluster of pain symptoms may be thought of as endometriosis-associated pain (EAP) (Morotti *et al* 2014).	True/False
9.	Endometriosis is a highly variable condition, yet many women with significant endometriosis will experience some form of pelvic pain (Fraser 2008).	True/False
10.	Matalliotakis (2008) reported a 21.6-fold increase in the risk of endometriosis among sisters and a 4-fold increase among mothers of those diagnosed with endometriosis.	True/False

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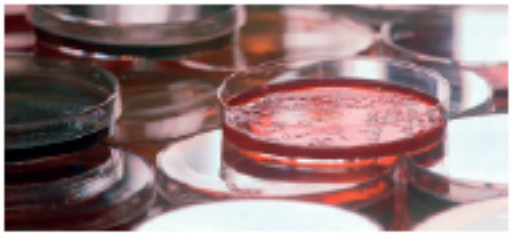

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1. **Bifidobacteria: Genomics & Molecular Aspects** edited by B. Mayo, & D. Van Sinderen. Caister Academic Press. xii + 260 pages.

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

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

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

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

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

13. **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.

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

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

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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

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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.

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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
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yr	year

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

Style Manual Committee. Council of Biology Editors. *Scientific style and format: the CBE manual for authors, editors, and publishers.* 6th ed. Cambridge University Press, 1994.

Style manual for authors, editors and printers. 6th ed. John Wiley & Sons Australia Ltd, 2002.

O'Connor M, Woodford FP. *Writing scientific papers in English: an ELSE-Ciba Foundation guide for authors.* Amsterdam, Oxford, New York: Elsevier-Excerpta Medica, 1975.

Day RA. *How to write and publish a scientific paper.* Philadelphia, Institute for Scientific Information Press, 1979.

Zeiger M. *Essentials of writing biomedical research papers*. 2nd ed. New York, McGraw-Hill, 2000.

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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