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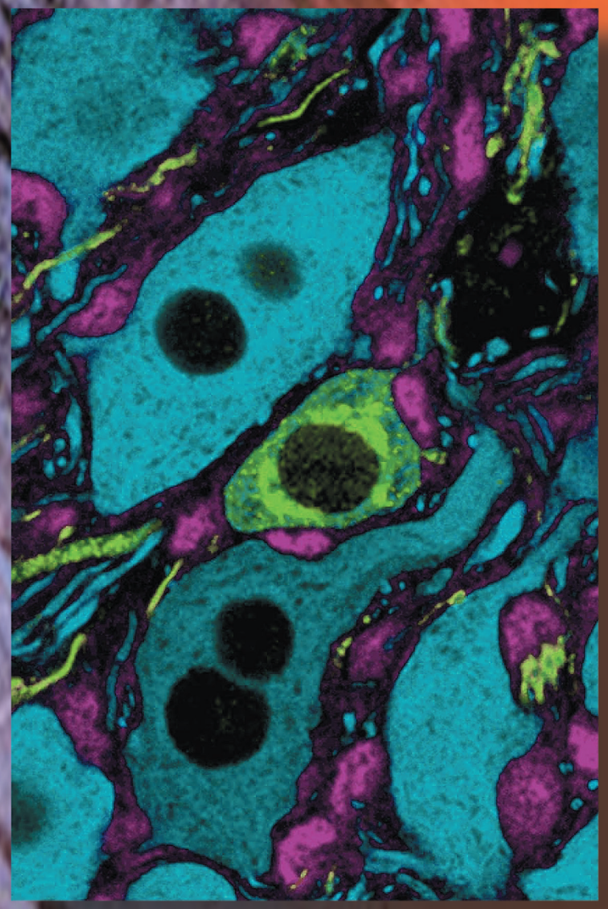

ORIGINAL ARTICLES

A validation of at-home infant stoole sample collection devices for determining the faecal microbiome

Dilute hydrogen peroxide: an alternative bleaching method for the Masson Fontana silver stain

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CONTENTS

February 2023 Vol. 44 No. 1

Original articles

A validation of at-home infant stoole sample collection devices for determining the faecal microbiome 2
Jacob A. F. Westaway, Roger Huerlimann, Yoga Kandasamy, Catheine M. Miller, Robert Norton, Donna Rudd

Dilute hydrogen peroxide: an alternative bleaching method for the Masson Fontana silver stain 11
Maria Boyer

Telehealth in the management of diabetes in regional and remote First Nations populations in Australia: where are the gaps? 20
Brenda Tseng and Thiru Vanniasinkam

Case Study

Juvenile myelomonocytic leukaemia case study 27
Hyeon Jeong Min

Regular Features

Journal-based CPD No. 88 33
Journal-based CPD No. 89 34
Book review 36
Instructions to authors 38
Fellowship of AIMS 42
Australian Council for the Certification of the Medical Laboratory Scientific Workforce 43

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

A validation of at-home infant stool sample collection devices for determining the faecal microbiome

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Abstract

In this study we present a validation of an at-home infant stool sample collection kit, the OMNIgene® GUT, for determining the faecal microbiome for use in longitudinal studies. Although developments in metagenomics and sequencing technologies have resulted in an explosion in microbiome research, studies exploring the long-term effects of probiotic modulation in infants are still relatively rare due in part to the challenges in the metagenomics methodology and standardised collection and storage techniques. To investigate the efficacy of the OMNIgene® GUT on microbial composition, we compared samples stored using the OMNIgene® GUT kit to the same samples collected in a standard/sterile collection tube and stored at -80°C for 3 months prior to 16S rRNA metabarcoding. No significant differences in read depth or microbial composition of samples were found between the two methods of collection and storage. When coupled with clear guidelines for self-collection and shipping, the OMNIgene® GUT kit is a feasible option for sampling preterm infants at home.

Keywords: ***Faecal, microbiome, sample storage, infants***

Introduction

Developments in metagenomics and sequencing technologies have been a catalyst for an explosion in microbiome research. This increase in research has led to an understanding of the importance of preterm infant gut microbiome development, and its emergence as a modifiable factor in neonatal intensive care (Westaway *et al* 2021a; Underwood *et al* 2009; Deshpande *et al* 2011). The role of the gut microbiome in metabolic and immune system development means that positive modulation of bacterial populations in the gut could provide long term metabolic and immune benefits. This is especially important for those infants born preterm where an immature gut microbiome has been linked to disease. A dysbiotic microbiome puts preterm infants at a greater risk of both acute diseases, such as necrotising enterocolitis (NEC) and sepsis (Collins *et al* 2018; Sharma and Hudak 2013), and chronic diseases, such as asthma and diabetes (Zhang *et al* 2018; Crump *et al* 2020). The introduction of probiotic supplementation has been shown to alleviate some of this disease burden (Sawh *et al* 2016; Thomas *et al* 2017) potentially through microbiome modulation (Alcon-Giner *et al* 2020; Abdulkadir *et al* 2016). Although this probiotic-associated microbiome modulation has been well characterised during their hospital stay, studies exploring the long-term effects of probiotic modulation are still relatively rare. This sparseness is likely to be linked to challenges in the metagenomics methodology.

Metagenomic studies investigating the microbiome are complex, technically challenging, and vary between laboratories. Each stage of the project protocol can

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introduce biases that influence outcomes (Lozupone *et al* 2013; Mao *et al* 2012) and contribute to heterogeneity between studies (Westaway *et al* 2021b). Arguably the most important pre-analytical consideration for faecal sampling is storage conditions prior to analysis. This is particularly important during point-of-care or at home collection, where freezing is not always an option, and the protocol involves untrained individuals. The sample collection methods therefore need to combine a simple method with robust storage. The reliability of downstream metagenomics analyses is highly dependent on appropriate storage conditions as this can influence both the stability of DNA and composition of the microbial communities (Cardona *et al* 2012).

Inadequate storage protocols may promote growth of specific taxa and can lead to DNA/RNA fragmentation in less than 24 hours at room temperature (Cardona *et al* 2012; Carroll *et al* 2012). Commonly available storage methods include freezing or refrigeration and the use of stabilizing buffers with the optimal method dependent on the duration of storage (Westaway *et al* 2021b). Freezing at -80°C is considered optimal for long-term storage, as it has been demonstrated to consistently yield microbiota composition closely related to that of fresh samples (Carroll *et al* 2012; Hale *et al* 2015). However, when immediate freezing is not logistically possible, storing samples in a preservation buffer is preferred as this can preserve genetic integrity for several weeks (Nechvatal *et al* 2008; Han *et al* 2018). Chemical storage is not without its issues though as these storage buffers may result in lower diversity (Menke *et al* 2017; Hill *et al* 2016) and some have been shown to impede downstream DNA extraction and amplification (Nechvatal *et al* 2008). These concerns may also be product specific.

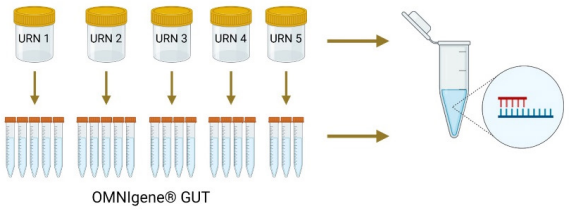
The objective of this project was to explore the performance of the OMNIgene® GUT kit in preserving microbial communities during at home collection. The OMNIgene® GUT kit is an all-in-one system for self-collection and stabilisation of faecal samples containing microbial DNA (DNA Genotek Inc. 2019). The kit includes a collection tube with 200µl of stabilising liquid. The sample is deposited into the tube and mixed with the stabilising liquid by shaking. This pilot study compares storage of preterm infant stool samples with the OMNIgene® GUT kit against storage in sterile collection kits at -80°C to determine if at home sample collection for future preterm infant studies using the commercial kit is a feasible protocol.

Method

Study Design

To investigate the effect of the OMNIgene® GUT on microbial composition, we compared samples stored using the collection kit to the same samples collected in a standard/sterile collection tube at -80°C for 3 months. We chose a subset of samples (n = 5) from a previous study, and aliquoted them into OMNIgene® GUT kits (OM-200 DNA Genotek Canada). Each sample had random proportions of stool added to mimic at home collection. The number of kits that were used for each sample was dependent on the amount of stool in the initial collection and the initial sample was used as the base level for the comparison. This resulted in a total of 26 technical replicates (21 stored with OMNIgene® GUT) from 5 different biological replicates (Figure 1).

A



B

Biological replicates	Technical replicates	
URN	Original sample	OMNIgene® GUT
1	1	5
2	1	5
3	1	4
4	1	4
5	1	3

Figure 1. *A visual description of the sampling method, where five original biological replicates were used to create 26 technical replicates by aliquoting the original replicates into OMNIgene® GUT collection kits.*

B. A table describing the number of original replicates and resulting technical replicates that were used for the analyses.

As part of the previous study, samples were immediately frozen and stored for ~12 months and then subsequently thawed for DNA extraction. A limitation of this study is that samples had been through one previous freeze-thaw cycle. The biological replicates are designated by the variable URN (unit record number) which has been de-identified from the original URN.

16S rRNA short amplicon sequencing

The Bioline ISOLATE Fecal DNA Kit (Bio-52038, Bioline Australia) was used for DNA extraction (Meridian 2020) which involves mechanical lysis, with modifications made in consultation with the manufacturer to optimise DNA yield. Modifications included increased beta-mercaptoethanol from 0.5 to 1% (increasing DNA solubility and reducing secondary structure formation), addition of an extra wash step (improving purity) and decreased elution buffer volume from 100µl to 50µl (increasing final DNA concentration). Additionally, a modification was made for compatibility with OMNIgene® GUT. After consultation with the manufacturer, a volume of 150 µl was chosen for the initial sample volume, with 90-150 mg used for the original samples. All previously listed modifications were applied to both sample types.

The Illumina metagenomics library preparation protocol was used for library preparation (Illumina Inc 2018) using the Index Kit v2 C (Illumina Inc 2020), along with Platinum™ SuperFi™ PCR Master Mix (ThermoFisher Scientific 2020). 16S rRNA short amplicon sequencing was performed on the Illumina MiSeq system using the MiSeq Reagent Kit V3 (Illumina Inc 2020) targeting the V3 and V4 regions with the S-D-Bact-0431-b-S-17/S-D-Bact-0785-a-A-21primer combination (Illumina Inc 2018). Pre-analytical bioinformatics were conducted in R Studio Version 3.6.1 (RStudio Team 2020) with a pipeline adapted from our previous work (Westaway *et al* 2021a), which can be found in the supplementary material. DADA2 (Callahan *et al* 2016) was used for quality filtering and trimming, demultiplexing, denoising and taxonomic assignment (using the SILVA Database), and the microDecon package (McKnight *et al* 2019a) was used to remove homogenous contamination from samples using blanks originating in extraction. The OMNIgene and original samples were processed from extraction through to analysis in parallel.

Statistical analysis

To compare storage methods, whilst accounting for the biological replicates, we assessed read depth, beta diversity, alpha diversity, and taxonomic abundance using mixed effects models. For beta diversity comparisons we performed both a PERMANOVA and an EnvFit analysis from the Vegan package (Dixon 2003), which compare the differences in the centroids relative to total variation. Both analyses were applied to a Bray-Curtis dissimilarity matrix (Jari Oksanen 2020) based on data normalised through Total Sum Scaling (TSS) (McKnight *et al* 2019b). The significance was based on 10,000 permutations and was transformed using the Benjamini-Hochberg (BH) procedure (Benjamini and Hochberg 1995).

For both alpha diversity and read depth we performed generalised linear mixed effects models using the package lme4 (Bates *et al* 2015). For diversity, both richness and the Shannon Index were calculated at the ASV level. Multicollinearity was assessed using the AED package (Luštrik and Stachelek 2009) and significance was determined using an analysis of deviance (Type II Wald Chi-square test) from the car package (Fox and Weisberg 2019). This was followed by subsequent post-hoc pairwise Tukey comparisons, to correct for multiple comparisons, using the emmeans package (Searle *et al* 1980).

DESeq2 (Love *et al* 2014) which uses a negative binomial generalized linear model and variance stabilising transformation was used for comparing taxonomic abundances between groups. Taxa were agglomerated at the genus level, due to the limited taxonomic depth of 16S-target technologies. A Wald Test with the BH multiple inference correction was performed to obtain taxa that were significantly differentially abundant among groups of interest. The pre-analytical bioinformatics and statistical analyses can be found in the GitHub link in the supplementary material.

Results

There was no significant different between storage with the OMNIgene® GUT collection kit and storage in sterile collection kits at -80°C. The OMNIgene® GUT collection kit did not have a significant effect on read depth (Figure 2) or microbial composition (Figs 3-5). The results of the analyses show that the samples clustered by the biological replicates (URN) rather than storage type. URN had a significant association with overall community composition (Figure 3A) and explained most of the variation/similarity between samples (Figure 3B) as demonstrated by both PERMANVOA ($p < 0.05$) and envfit ($p < 0.05$) mixed effects models. Despite some variation as shown in Figure 4, the storage method also had no significant effect on alpha diversity (Shannon Index: $p = 0.19$, Richness: $p = 0.74$). Lastly, when exploring taxonomic abundance, it did appear that the original sample had differences in taxonomy relative to those from the OMNIgene® GUT collection kit. Differential abundance testing however with DESeq2 demonstrated that despite this difference that overal, the storage method had no significant effect on the abundance of taxa (Figure 4). Additionally, despite this variation in taxonomy between sample storage within URN 4, these samples still clustered based on taxonomic composition (Figure 3A).

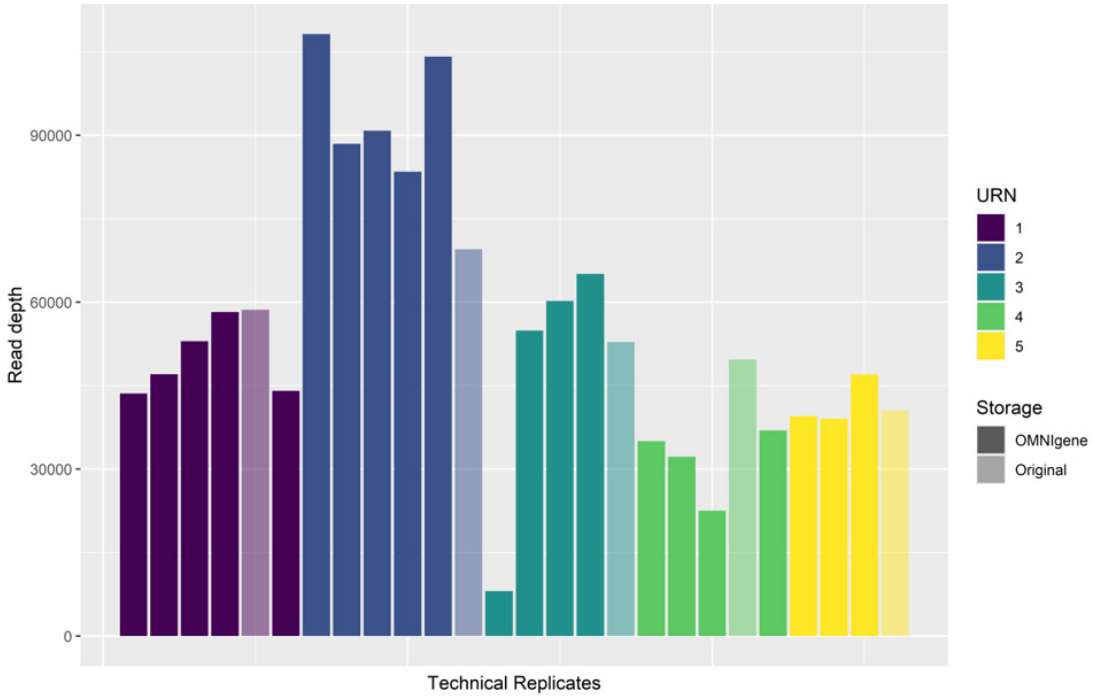
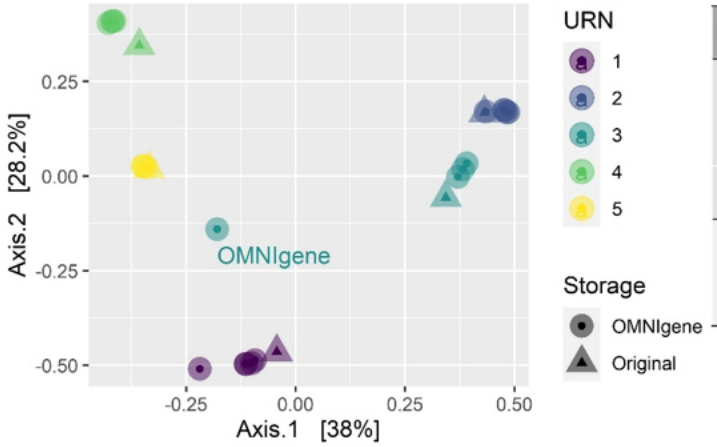


Figure 2. *Column graph illustrating the similarities in read depth between technical replicates and coloured by biological replicates. Storage method had no significant effect on read depth (p = 0.87).*

A



B

Analysis	Variable	r2	Adjusted-p
PERMANOVA	Storage	0.88	<0.01
	URN	<0.01	0.28
Homogeneity condition			0.90
envfit	Storage	<0.01	0.92
	URN	0.93	<0.01

Figure 3. *A. Principal coordinate analysis plot based on Bray-Curtis distances describing the similarity/dissimilarity of samples based on taxonomic composition and demonstrating the clustering of samples based on URN (p < 0.01) and not the storage method (p = 0.24), with the exception of a single outlier (designated OMNIgene).*

B. Table describing the results from both a PERMANOVA performed with adonis2 (and the subsequent test for homogeneity of variance) and an envfit analysis performed on Bray-Curtis distances.

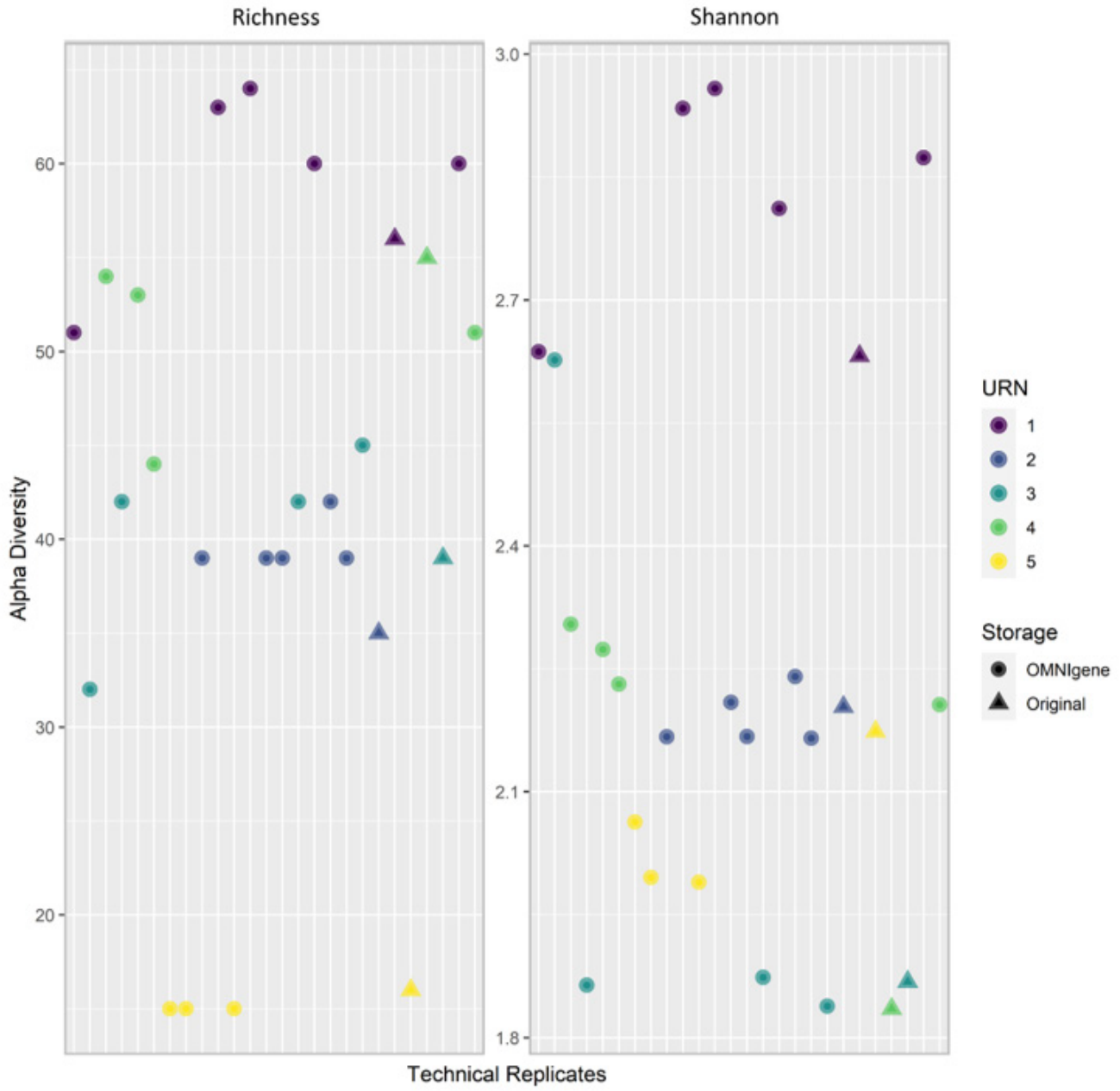


Figure 4. *Dot plots, coloured by the URN and faceted by alpha diversity measure, representing the distribution of alpha diversity metrics across samples in the storage comparison. Alpha diversity was significantly associated with URN (Shannon: $p < 0.001$ & richness: $p < 0.001$) and not sample storage (Shannon: $p = 0.19$ & richness: $p = 0.74$).*

Using the OMNIgene®GUT collection kit is a viable option for at home collection when immediately freezing at -80°C is not possible.

Discussion

Optimal storage conditions are a key starting point of pre-analytical variation for all metagenomics studies. Without optimal storage conditions, samples may be compromised and any conclusions drawn from the data unreliable. Previous work has found that storage on ice for up to 48 hours (Wu *et al* 2010), or 4°C for 24 hours (Tedjo *et al* 2015) is sufficient if samples are to be processed immediately. For long-term storage however, freezing at -80°C is best as it inhibits bacterial growth and/or degradation, as demonstrated by similarities between long term storage of faecal samples at -80°C and fresh samples (Carroll *et al* 2012; Hale *et al* 2015; Shaw *et al* 2016). It is therefore common for samples to be stored at -20°C or 4°C until freezing at lower temperatures is possible. Alternatively, preservation buffers, like DNA/RNA Shield (Zymo Research) and RNAlater (Thermofisher),

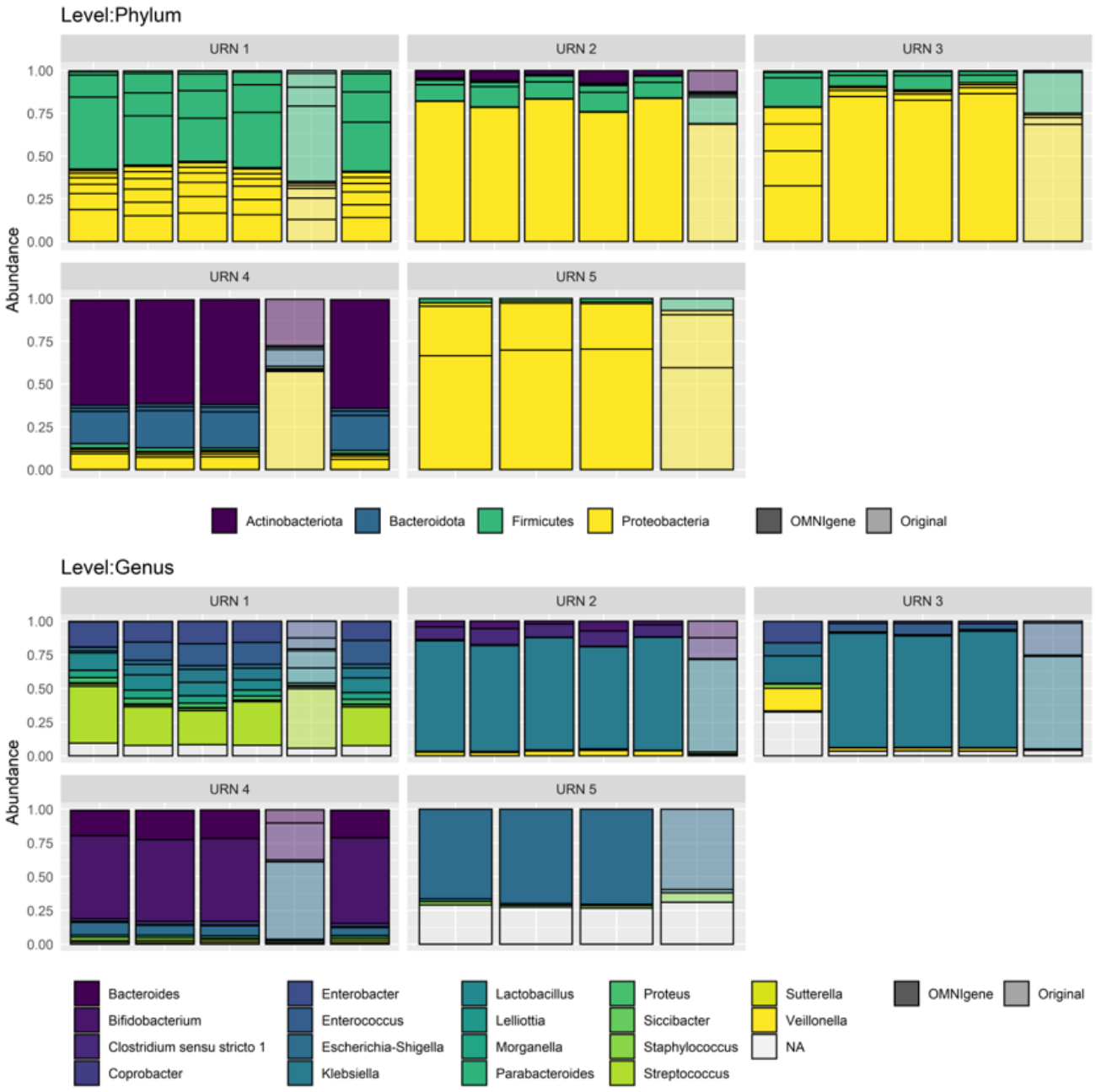


Figure 5. *Bar plots comparing the distribution of the top 20 most abundant genera (colours) across technical replicates (individual bars), and within URN (facets) for the storage method comparison.*

have been shown to preserve genetic integrity for weeks without refrigeration or freezing (Nechvatal *et al* 2008; Han *et al* 2018; Anderson *et al* 2016; Song *et al* 2016; Choo *et al* 2015; Flores *et al* 2015; Dominianni *et al* 2014). Some preservation buffers however may result in lower diversity or impede downstream DNA extraction and amplification of target variable regions (Nechvatal *et al* 2008). The objective of this study was to determine if storage with OMNIgene® GUT kit had a significant effect on metagenomic outcomes and is a feasible option for at home sample collection.

The DNA obtained from samples stored in the OMNIgene® GUT was sufficient for DNA extraction, amplification of variable regions and sequencing. The adequacy of the DNA extract can be assessed by estimating the read depth captured during sequencing. Sufficient read depth is necessary for capturing an accurate representation of microbiome composition and for producing accurate diversity metrics (McKnight *et al* 2019b). Our results show that there is no significant variability between technical replicates belonging to the same biological replicate. The only outlier is a technical replicate within URN 3 with

low read depth, which is also evident in the PCoA. It is unclear as to why this sample has low read depth relative to the other technical replicates. This could be due to an error in the aliquoting or library preparation protocol. Importantly, this sample was from an OMNIgene® GUT kit but the other three technical replicates stored in the same manner within this URN are relatively homogenous.

The similarities in read depth between storage methods translated into similarities in microbial composition. Similar to previous work by Hill *et al* (Hill *et al* 2016) our samples clustered significantly by the biological replicate, suggesting that the storage method has no effect on microbial composition, despite some taxonomic variation in URN 4. Other studies have demonstrated deficiencies in some preservation treatments can result in a reduction in alpha diversity and particular taxa, however, the microbial diversity and composition produced by the OMNIgene® GUT kit was similar to storage in a standard collection jar at -80°C. Despite the reliability of non-freezing preservation methods being called into question in the past (Menke *et al* 2017; Hill *et al* 2016; Nechvatal *et al* 2008) the counter narrative presented in this study is likely to be an effect of different products producing different results, which has also been noted previously (Menke *et al* 2017).

The present study shows that the OMNIgene® GUT kit is a reliable and repeatable storage option for faecal microbiome studies. This supports results from a recent study by Szopinska *et al* (2018) who concluded that the OMNIgene® GUT kit is a participant-friendly collection method (Szopinska *et al* 2018). Not only is the kit reliable in its preservation of microbial communities, it is coupled with clear guidelines for self-collection (DNA Genotek Inc. 2019). The OMNIgene® GUT kit has no deleterious effects on preservation of microbial communities and when coupled with clear guidelines for self-collection and shipping, is a feasible option for sampling preterm infants at home.

Conclusion

The current study demonstrates that the OMNIgene® GUT kit is an easy to use and robust and repeatable method for at-home sample collection when immediate freezing is not possible. The kit produces microbial populations that do not deviate significantly from what is considered the gold standard for long term storage. Using this method would allow for at-home sampling as part of an investigation into the gut microbiome of preterm infant, post-discharge. These findings would also be translatable and support at-home sampling with the OMNIgene® GUT kit for other microbiome studies using human stool samples.

Acknowledgements

Figure 1 was created with Biorender.com.

Statement of financial support

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Consent Statement

The research was performed in accordance with the Declaration of Helsinki and ethics approval was obtained from the Human Research Ethics Committee from the Townsville Hospital and Health Service (HREC/QTHS/65181).

Availability of data and materials

The analysis workflow, both the bioinformatics pipeline and statistical analyses, can be found at JacobAFW/Long_term_effects_of_probiotics (github.com)).

To avoid duplication online the sequencing dataset analysed during the current study are not available online. However, the data can be provided upon request to the corresponding author.

Conflict of Interest

This study was undertaken as part of the requirement for PhD studies and the work was partially funded by a Doctor of Philosophy (PhD) Research Seeding Grant provided through the AIMS Research Engagement scheme. The second author (DR) is currently a member of the AIMS Board.

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Dilute hydrogen peroxide: an alternative bleaching method for the Masson Fontana silver stain

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Abstract

Melanin is a structurally complex brown-black pigment found in living organisms across multiple biological kingdoms including plants, animals, and microorganisms. The pigment is insoluble in organic solvents and can cause significant complications in the histopathological and molecular diagnosis of heavily pigmented melanocytic lesions. Dense melanin pigment obscures cellular morphology and physically masks antigen-antibody interactions. Melanin is susceptible to bleaching with strong oxidants such as potassium permanganate and hydrogen peroxide. The Masson Fontana silver stain for the detection of melanin pigment incorporates a control slide that is traditionally bleached with potassium permanganate to confirm the presence of melanin in an unbleached test section. This experiment uses hydrogen peroxide in place of potassium permanganate. The results demonstrated hydrogen peroxide to be a superior bleaching agent with improved tissue morphology and adhesion properties.

Keywords: ***Melanin pigment, hydrogen peroxide, bleaching, melanoma, Masson Fontana***

Introduction

Melanins are high molecular weight complex pigments found across all living kingdoms, and display great diversity in structure, colour, density, and function (Solano 2014). Melanin is tightly bound to proteins within the melanosome making the pigment extremely resistant and completely insoluble in most organic solvents (Suvarna *et al* 2019). Structurally, there are two chemically distinct types of melanins found in human tissue, identified as eumelanins and pheomelanins. Eumelanins are the most common type of melanin and consist primarily of 5,6 dihydroxyindole units. Eumelanins provide the dark brown to black pigment to hair, skin, and the iris of the eye. Pheomelanins chemically comprise of sulfur and cysteine containing units referred to as benzothiazine and benzothiazole (Pina-Oviedo *et al* 2014).

Pheomelanins produce yellow and red coloured pigment and are found in red hair, freckles and the skin of lips and genitals. Neuromelanins, the black pigment observed in the catecholaminergic neurons of the substantia nigra and locus coeruleus of the mid brain chemically consists of a mixture of eumelanin and pheomelanin (Haining and Achet-Mendes 2017).

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Melanomas demonstrate distinct pathological and molecular subtypes and KIT, BRAF and NRAS mutations have been found in a percentage of melanoma cases. Molecular diagnosis of melanomas is clinically significant as it may identify patients suitable for targeted therapies with BRAF, MEK and or KIT inhibitors (Namikawa and Yamazaki 2019). Melanin pigment has the capacity to absorb a wide range of ultraviolet radiation, which can interfere with the accurate photometric quantification of nucleic acids. Furthermore, the pigment can also bind to DNA polymerase, hindering the amplification and isolation of nucleic acids in polymerase chain reaction based molecular assays (Chung *et al* 2016).

The physical masking effects of melanin pigment can impact the histological and molecular diagnosis of melanocytic lesions (Chung *et al* 2016; Hu *et al* 2020). Traditional bleaching methods incorporating the use of strong oxidants such as the combined potassium permanganate oxalic acid method have been successful in eliminating the masking effects of the pigment with limited to complete loss of antigenicity (Orchard 1999) and nucleic acids. In contrast, using dilute, heated solutions of hydrogen peroxide has been successful in removing the pigment whilst preserving cell antigenicity and nucleic acids, critical for the diagnosis and accurate classification of melanocytic lesions. Although there is great variability

in the literature of the optimal conditions, this method of bleaching has been demonstrated by various studies (Momose *et al* 2011; Chung *et al* 2016; Orchard *et al* 2019) as the preferred method for immunohistochemical and molecular testing.

The Masson Fontana is a silver staining technique widely used for the demonstration, localisation, and quantification of melanin in skin pigmentation and disease. A bleaching method is incorporated into Masson Fontana staining methods to confirm the presence of melanin in the unbleached test section, a valuable technique used in the diagnosis of malignant melanoma. In our laboratory the use of potassium permanganate has been problematic with disruptions to the morphology and at times complete loss of test sections in prolonged incubations.

This study was conducted to determine if hydrogen peroxide is a superior bleaching agent compared to the potassium permanganate - oxalic acid method currently used in the Masson Fontana protocol. The experiment would also identify the optimal conditions to ensure effective, reproducible, and consistent bleaching, taking into consideration the heterogenous nature of melanin pigments and the various tissue types diagnosed within the laboratory.

Materials and Methods

Tissue samples

Testing was performed in multiple phases with adjustments and refinements made to incubation times and temperatures.

The study cohort consisted of a total of 14 cases of formalin fixed paraffin embedded (FFPE) tissue sections containing melanin pigment. Test sections of nasal mucosa, skin excision and punch biopsy were sampled in duplicate for each phase of the testing. At phase three additional tissue types including brain, liver core biopsy, appendix, uterus, lymph node and ileum were introduced to test a wider range of specimen types. All tissue samples were cut at 3micron thickness and collected onto Series 3 (Trajan Australia) and Superfrost Plus (Thermo Scientific Australia) adhesive microscopic glass slides. The sections were baked and deparaffinised on the Roche HE600 autostainer (Roche Ventana, Medical systems USA) prior to bleaching and staining. The optimised protocol required baking the FFPE sections in an 80°C oven for 15 minutes prior to deparaffinization in the HE600 autostainer.

Bleaching

The bleaching step was performed using a 30% hydrogen peroxide (H2O2) stock solution which was diluted to 3%, 5%, 10% and 15% concentrations with phosphate buffered saline pH 7.4. The working solutions were made to a total volume of 50 mL, placed in glass Coplin jars and heated to the required temperatures in a temperature-controlled water bath.

The bleaching was performed over five experimental phases to determine the optimal conditions for bleaching efficacy and reproducibility. Table 1 indicates the temperature and incubation times analysed. At the completion of the bleaching process, all sections were rinsed thoroughly with distilled water prior to staining in the Masson Fontana silver nitrate solution.

***Table 1**. Demonstrates the temperature and incubation times analysed for the bleaching process.*

Phase 1			
H_2O_2 Dilution	Temperature	Incubation time	
5% and 10% H_2O_2	60°C	30 minutes	60 minutes
15% H_2O_2	60°C	30 minutes	
Phase 2			
3%, 5% and 10% H_2O_2	55°C	60 minutes	
Phase 3			
5% and 10% H_2O_2	45°C	60 minutes	120 minutes
Phase 4			
10% H_2O_2	45°C	60 minutes	
Phase 5			
10% H_2O_2	45°C	90 minutes	

Masson Fontana silver stain

The Masson Fontana (MF) was performed using a fresh ammoniacal silver nitrate working solution. An unbleached control slide for each test issue was run parallel with the bleached section to assess efficacy.

The ammoniacal silver working solution was made using 20 mL of 10% silver nitrate, and 25% ammonia stock solution which was added drop by drop until the precipitate almost dissolved and the solution appeared opalescent. An additional 20 mL of distilled water was added, and the solution was filtered into a glass Goplin jar and heated to 60°C in a temperature-controlled oven.

The test and control sections were incubated in the silver nitrate working solution at 60°C for 25 minutes. The unbleached control sections were checked microscopically to ensure the melanin pigment was intensely black. The sections were rinsed thoroughly with distilled water and treated with 2% sodium thiosulphate for 5 minutes to remove unreacted silver ions. Sections were rinsed with distilled water and counterstained. The slides were rinsed, dehydrated and coverslipped. Bleaching efficacy was assessed microscopically sing bright field microscopy with x100 magnification.

Results

Determination of optimal H_2O_2 dilution for complete depigmentation

Phases 1 and 2 were assessed using test sections that contained the darkest and most abundant pigment, a nodular melanoma (scalp lesion) and a sinonasal melanoma. Complete depigmentation was achieved at 5% and 10% H_2O_2 at 60°C after a 60-minute incubation. The structural integrity of the tissue was disrupted with increasing concentration and incubation of hydrogen peroxide. The intensity of the neutral red counterstain was remarkably reduced in sections that were bleached for 60 minutes making it difficult to assess the morphology.

Phase 2 of the experiment determined that a 5% H_2O_2 dilution was not effective at removing the pigment to completion in the sinonasal melanoma after a 60-minute incubation when the temperature was reduced to 55°C. Prolonged incubations (120 minutes) using the 5% H_2O_2 dilution completely removed the pigment in the nasal melanoma but resulted in complete loss of the liver core and skin punch biopsy in phase 3 of the testing. The optimal dilution to achieve complete depigmentation within a 60-minute incubation was 10% hydrogen peroxide solution.

Determination of optimal temperature and incubation time to achieve complete bleaching

Complete bleaching was achieved at 60 minutes with dilutions of 5% and 10% hydrogen peroxide at 60°C. Loss of structural preservation of tissue sections was noted with all dilutions of hydrogen peroxide at 60°C. Reducing the temperature to 55°C only slightly improved the preservation of the morphology and tissue adherence of larger tissue sections, however only the 10% hydrogen peroxide solution was successful in removing the pigment at this temperature within 60 minutes. Heating the 10% H_2O_2 solution to a maximum of 45°C proved to be the ideal compromise between bleaching efficacy and morphological preservation when the sections were incubated for 60 minutes. Brain, lymph node, uterus, appendix, ileum and liver core tissue were bleached using these conditions and the morphology was preserved with greater success than previous incubations performed at higher temperatures. Prolonged bleaching exceeding 60 minutes (Phase 5) increased the risk of section lifting and loss of structural integrity. The skin specimens appeared to be the most impacted with complete loss of the punch biopsy section post bleaching at 5% H_2O_2 incubated for 120 minutes. The scalp lesion was partially affected when bleached for 90 minutes using the optimal conditions. The structural integrity of the liver core, brain and lymph node was preserved when the sections were bleached for 90 minutes using the 10% H_2O_2 at 45°C.

Assessment of counterstains post bleaching

The original Masson Fontana protocol incorporates a 1% neutral red counterstain applied for 3 minutes. The intensity of the neutral red counterstain was significantly reduced in the bleached sections making it very difficult to assess the morphology. Applying the 1% neutral red solution for an extended 7 minutes and the 0.1% fast green applied for 3 minutes failed to make a considerable improvement to view the bleached sections. The ready to use Harris' haematoxylin applied for 3 minutes stained the bleached sections with the greatest intensity, however the contrast between the melanin pigment and the haematoxylin in the unbleached sections was inadequate, as viewed in the epidermis of the skin (Figure 3). The 0.1 % nuclear fast red solution applied for 5 minutes proved to be the most suitable counterstain, providing sufficient intensity to view the morphology in the bleached sections without obscuring the melanin pigment in the unbleached sections (Figure 4).

Discussion

The two most widely used bleaching methods for histological assessment of melanocytic lesions are the potassium permanganate oxalic acid and the dilute hydrogen peroxide methods. Both methods can be easily incorporated into staining protocols manually Orchard *et al* (2019) and Liu *et al* (2018) successfully implemented the dilute hydrogen peroxide method into automated

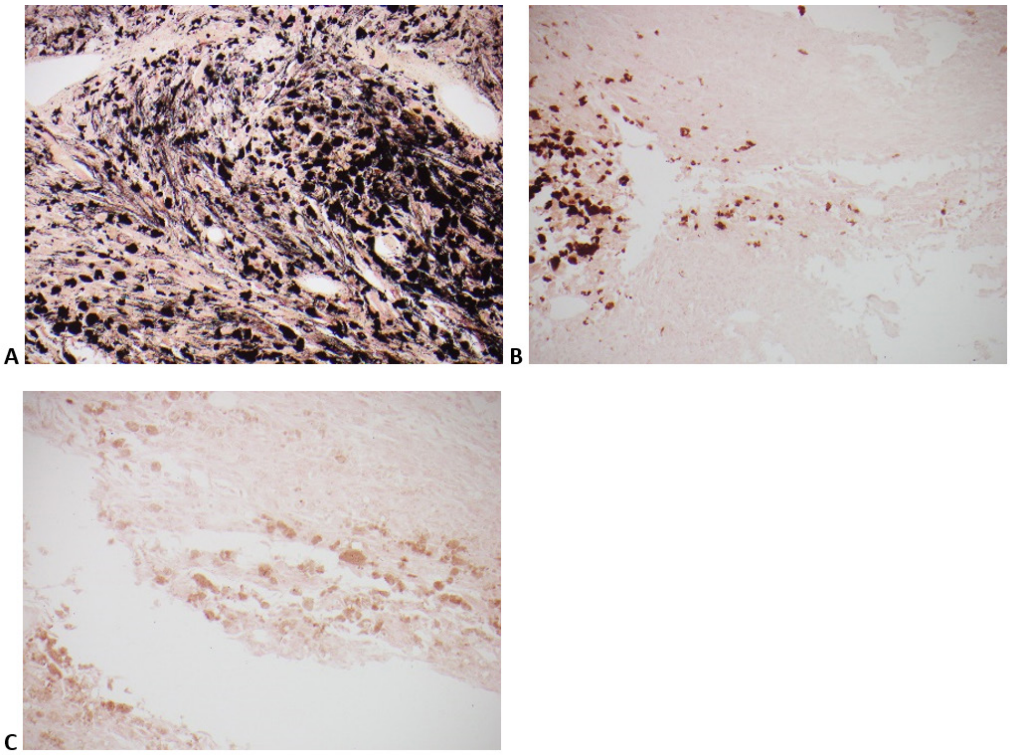

Figure 1. *Sinonasal melanoma. (A) Unbleached control section (MF x100). (B) Bleached with 5% H_2O_2, at 60°C for 30 minutes (MF x100). (C) Bleached with 15% H_2O_2 at 60°C for 30 minutes (MF x200). Sections were adhered to Trajan series 3 slides. Images (B) and (C) demonstrate incomplete depigmentation and minor loss of structural preservation.*

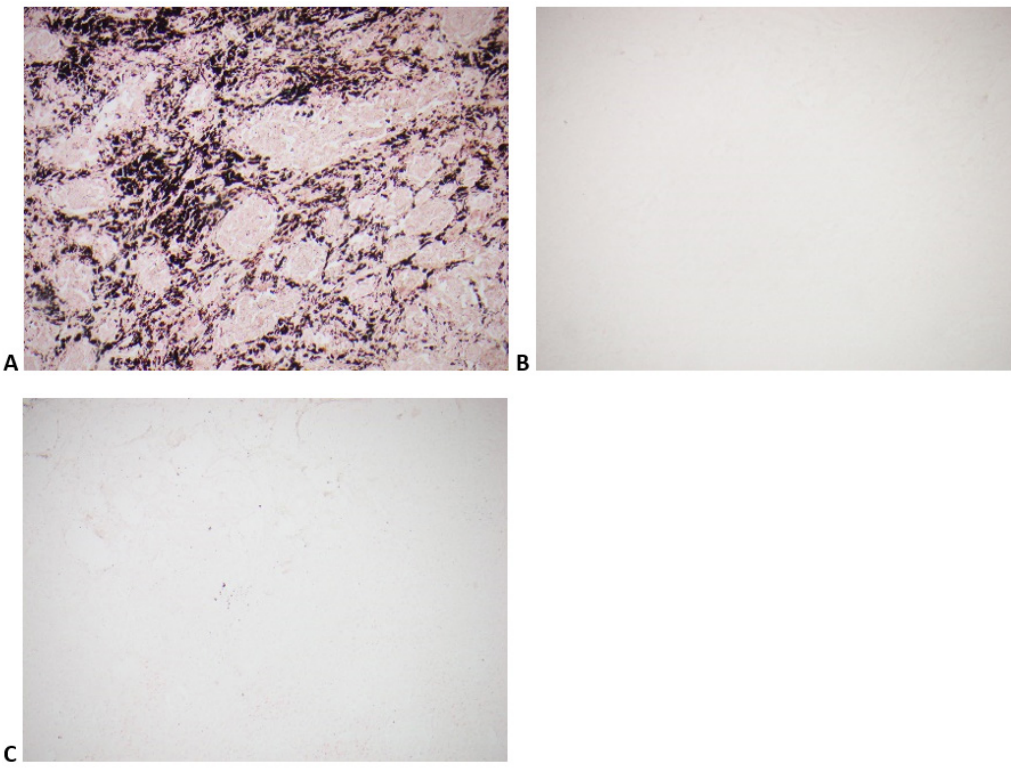

Figure 2. *Nodular melanoma Scalp Lesion. (A) Unbleached control section (MFx100). (B) Bleached with 5% H_2O_2 at 60°C for 60 minutes (MF x100). (C) Bleached with 10% H_2O_2 at 60°C for 60 minutes (MF x100). Sections were adhered to Trajan series 3 slides. Images (B) and (C) demonstrate complete bleaching, significant loss of structural preservation and weak counterstaining with neutral red (3 minutes).*

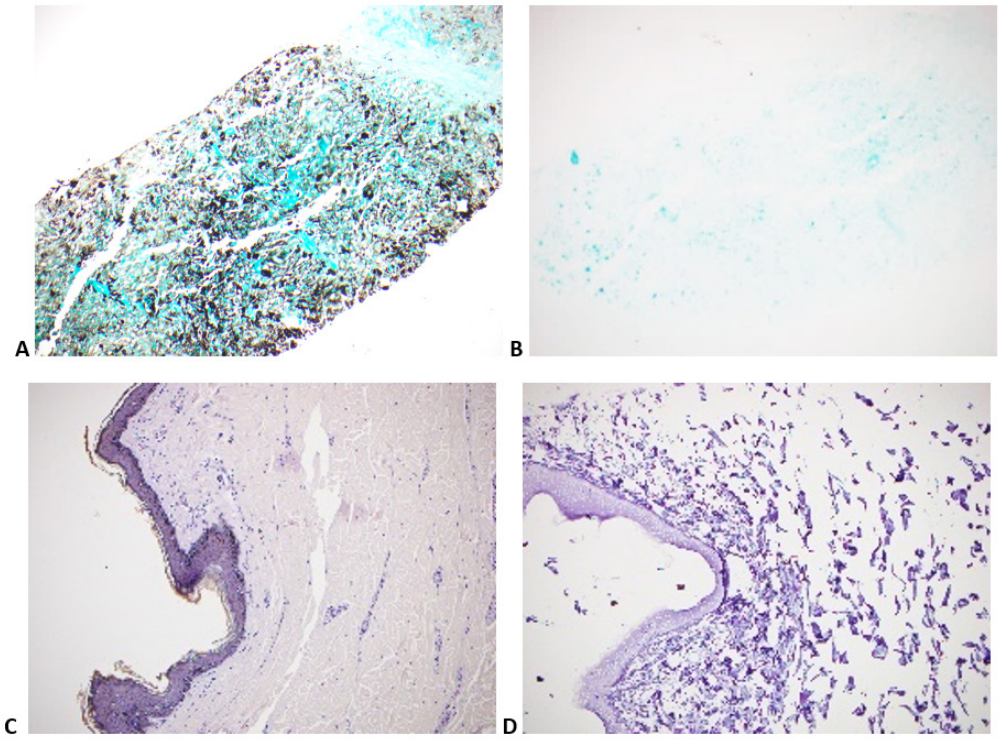

***Figure 3**. (A) Liver biopsy stained with Masson Fontana and 0.1% fast green counterstain. (B) Liver core bleached and stained with 0.1% fast green. (C) Skin excision stained with Masson Fontana and Harris' haematoxylin counterstain. (D) Skin excision bleached and counterstained with Harris' haematoxylin.*

immunohistochemistry platforms, the Roche ultra and Leica Bond respectively.

Both oxidants are effective at removing the melanin pigment but have their limitations, compromising the morphological detail and or antigenicity in prolonged bleaching steps.

Potassium permanganate is a very powerful oxidant as its high electron absorbing ability breaks the carbon-to-carbon bonds in proteins destabilising the protein whilst causing the bleaching effect (Hu *et al* 2020). Weak dilutions as low 0.25% are effective at removing the pigment more rapidly than the hydrogen peroxide, however its destabilising effect on proteins has a detrimental effect on immunohistochemical testing (Hu *et al* 2020; Orchard *et al* 2019; Shen and Wu 2015; Momose *et al* 2011).

In our laboratory, a fresh 0.25% solution of potassium permanganate ($KMnO_4$) followed by oxalic acid is the method currently in use for bleaching the melanin control in the Masson Fontana silver stain. The bleaching step is performed at room temperature for a minimum of 20 minutes. Some of the problems experienced using this method include incomplete bleaching (requiring extended bleaching incubations), section lifting and altered staining properties. Section lifting and disruption to the morphology particularly in prolonged bleaching steps requiring incubations greater than 30 minutes is the most significant problem encountered. Disruption to the morphology can prevent the ability to localise the pigment in lesions and accurately assess the pathology. In some instances, there has been complete loss of test sections resulting in repeat testing and unnecessary loss of patient test tissue and costly reagents.

This study assessed if using a heated solution of hydrogen peroxide (H_2O_2) to bleach melanin pigment is a superior alternative to the potassium permanganate oxalic acid method when performing a Masson Fontana stain.

A literature review of 10 articles that described bleaching protocols using dilute hydrogen peroxide on FFPE tissue revealed wide variability in testing conditions and bleaching efficacy. In summary, the hydrogen peroxide concentrations used in these protocols ranged from 0.5% (Chung *et al* 2016) to 30% dilutions (Hu *et al* 2020). The solutions were utilised at room temperature (Hu *et al* 2020) and heated to 50°C and 55°C (Momose *et al* 2011), 60°C (Jordan *et al* 2019: Momose *et al* 2011; Orchard 2007) 65°C (Liu *et al* 2013: Manicam *et al* 2014), and up to 80°C (Chung *et al* 2016). The incubation times ranged from 15 minutes to 24 hours for complete bleaching. In addition, some of the diluents used included distilled water, phosphate buffer, TRIS and phosphate buffered saline (PBS). Manicam *et al* (2014) determined that using PBS pH7.4 was a superior diluent compared

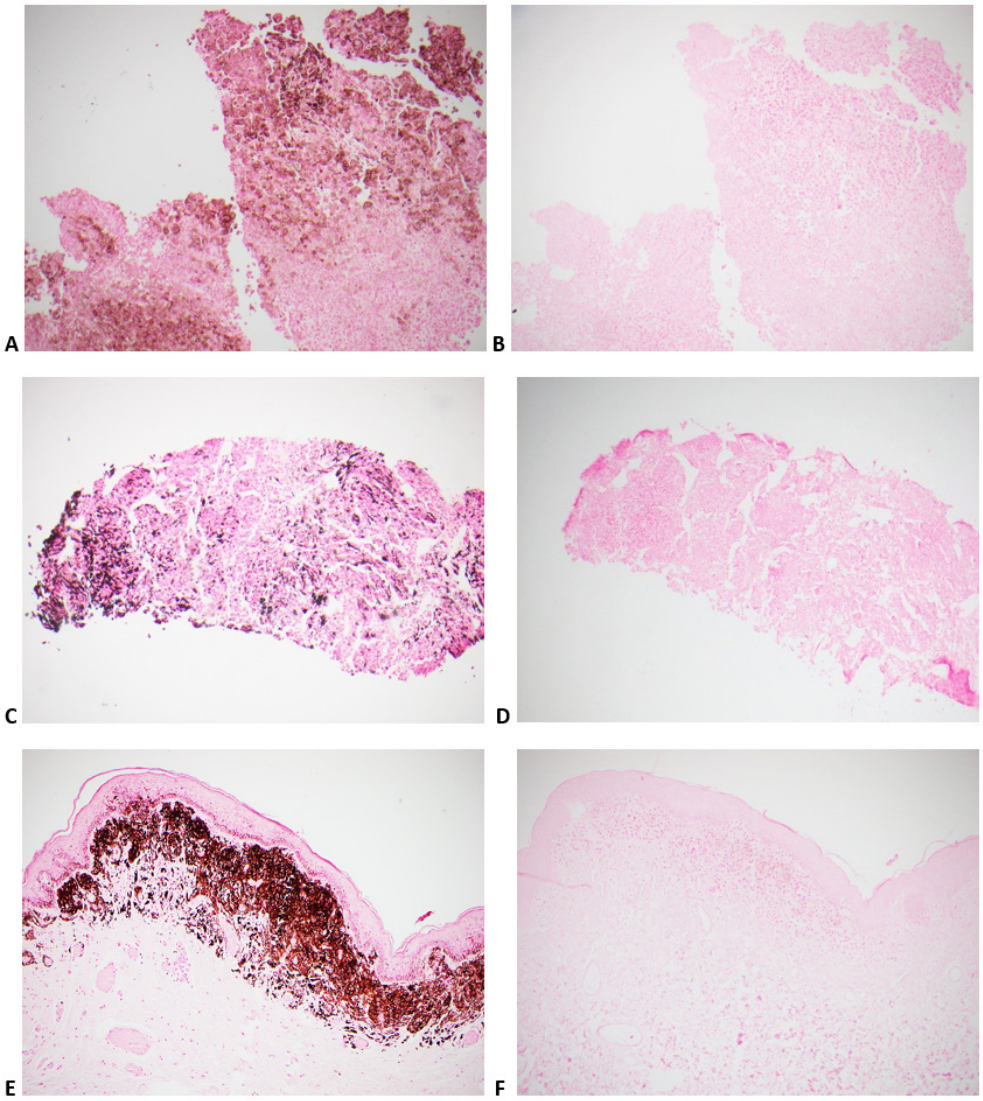

***Figure 4**. Sections bleached with 10% H_2O_2 at 45°C for 60 minutes, stained with Masson Fontana, counterstained with nuclear fast red. (A) Frontal brain tumour with metastatic melanoma unbleached control section (x100). (B) Frontal brain tumour bleached section (x100). (C) Liver core biopsy with metastatic melanoma unbleached (x100). (D) Liver core biopsy bleached section (x100). (E) Benign intradermal naevus unbleached control section (x200). (F) Benign intradermal naevus bleached section (x200).*

to distilled water in their investigations. The articles described these bleaching methods for applications in immunohistochemical and molecular testing.

To date the author has not found an article that documents a hydrogen peroxide bleaching protocol specifically applied to the Masson Fontana silver stain.

Orchard (2007) indicated that a 10% hydrogen peroxide solution is an effective concentration to achieve complete bleaching in most FFPE tissues within an hour when heated to 60°C. This was convenient to be able to perform the bleaching and the silver staining in the one waterbath set at a constant temperature as the silver stain in the Masson Fontana also requires the silver solution to be heated to 60°C. Unfortunately, the results of phase one demonstrated some degree of section detachment in all dilutions at 60°C post bleaching and prior to silver staining. Complete bleaching was achieved at 60 minutes for the 5% and 10% hydrogen peroxide solution. At a concentration of 10% and above however cytoplasmic detail of some parts of the tissue was disrupted, particularly the adipose component of the skin. This may be due to the compound effect of the bleaching followed by incubation of the tissue in a heated ammoniacal silver solution. The alkalinity of the silver solution does pose a risk of the tissue sections detaching during the staining process even when sections are adhered to adhesive slides.

The most remarkable observation noted at the end of phase one was how pale the neutral red counterstain appeared on the bleached sections. This made it difficult to assess the morphology and limited the ability to take

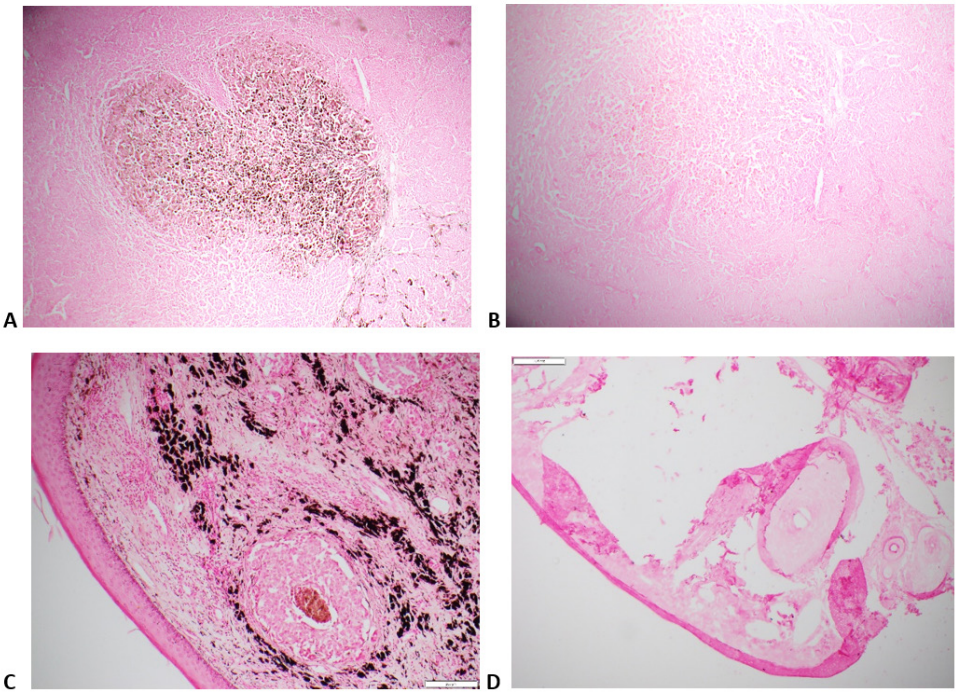

***Figure 5**. Prolonged bleaching with 10% H_2O_2 at 45°C for 90 minutes. (A) Lymph node (MF x100). (B) Lymph node bleached section (x100). (C) Scalp lesion (MF x200. (D) Scalp lesion, bleached section demonstrating significant lifting and disruption to the morphology (MF x200).*

microscopic images of these sections. This is not a problem with the current $KMnO_4$/oxalic acid method. The results of phase one indicated that the 60°C temperature was too high to perform the bleaching as this compromised the morphological preservation of the sections and the intensity of the counterstain. This finding was inconsistent with previous studies by Momose *et al* (2014), Manicam *et al* (2014) and Liu *et al* (2018) who stated that using 10% hydrogen peroxide solutions heated to 60°C and 65°C sustained morphological preservation even after incubation periods extending to 2 hours.

The results of phase two demonstrated that decreasing the temperature to 55°C only slightly improved section adherence of the larger tissue samples, however there was still some lifting noted with the 3 % and 5% dilution of H_2O_2 in the nasal melanoma sample and complete loss of a skin punch biopsy of a pigmented naevus. The 5% dilution heated to 55°C was sufficient to completely bleach the melanin pigment in the melanocytes of the skin sample but was not as effective at removing the pigment completely in the nasal melanoma. Phase two demonstrated that a heated 10% dilution of H_2O_2 was the most effective concentration to achieve complete bleaching in 60 minutes as documented in studies by Momose *et al* (2011), Liu *et al* (2013) and Orchard *et al* (2019).

Determining the optimal temperature required the most testing. All studies reviewed in the literature for this exercise indicate that using heated solutions of hydrogen peroxide reduce the bleaching time notably. Elevated temperatures however can compromise the preservation of structural integrity in tissue sections, as evident in this experiment.

A substantial reduction in temperature, to 45°C and baking the slides for an additional 15 minutes in an 80°C oven prior to deparaffinization made a significant improvement to section adherence without compromising the bleaching efficacy during a 60-minute incubation.

By the end of phase three, optimal key parameters were identified: the optimal concentration was 10% H_2O_2, the optimal temperature appeared to be 45°C and complete bleaching was observed at 60 minutes for our sample cohort. Additional tissue types including uterus, liver core, brain, ileum, and appendix were analysed to assess the morphological preservation of different tissue types following the bleaching process (Figure 5). Morphological preservation was noted in all the additional tissue types.

Assessment of the different counterstains revealed the 0.1% fast green and the neutral red still appeared faint and moderately hindered the ability to assess the pathological features in the bleached sections, as demonstrated in Figure 3B. With the intensity of the counterstain remarkably weak compared to the unbleached sections. This suggests that the oxidative properties of the hydrogen

peroxide impact the affinity of some counterstains, an observation not encountered with the $KMnO_4$/oxalic acid method. The Harris' haematoxylin stained the bleached sections with the greatest intensity compared to the other counterstains assessed in this study as evident in Figs 3C and D. This may be due to both Harris' haematoxylin and Nuclear Fast Red using aluminium mordants in their staining mechanisms.

The Masson Fontana silver stain is primarily used to detect and localise melanin pigment in disease, with the prime importance being the detection of metastatic melanoma. In some cases these deposits can be minimal and may be obscured if the counterstain is too intense and or does not provide adequate contrast. This study showed that the 0.1% nuclear fast red applied for 5 minutes (Figs 4 and 5) demonstrated the cellular detail in the bleached sections significantly better than the fast green and neutral red counterstains and provided superior contrast compared to the Harris' haematoxylin.

The final experimental phase of this study involved assessing the morphological preservation of test tissue following a prolonged bleaching step using the optimised bleaching conditions of 10% H_2O_2 heated to 45°C. Phase 5 assessed preservation of tissue morphology after 1.5 hours. This exercise was conducted to gain additional data that may improve the optimisation of the method should darker pigments require extended bleaching incubations. Melanin pigment is heterogenous in density and across different tissue types e.g. the eye comprises of several layers of melanin pigment often requiring longer bleaching times than non-ocular FFPE tissue (Manicam *et al* 2014). Our results demonstrated some variability with resilience to prolonged bleaching steps. Skin samples were the most susceptible to tissue disruption and the lymph node excision appeared the most resilient (Figure 5). This suggests that perhaps longer drying and baking times for skin sections may be necessary prior to bleaching and staining. Consideration to using alternative adhesive slides such as chromium gelatin subbed slides as used by Manicam *et al* (2014) is also worth noting.

Limitations of the study

This was a pilot study comprising of a total of 14 cases of FFPE tissue sections containing melanin pigment of variable densities and quantity. Ocular melanin was not assessed in this study. Further validation testing is required for skin punch biopsies as these samples continued to detach during the bleaching process throughout all the experimental phases and could not be assessed.

Conclusion

The optimised hydrogen peroxide protocol proved to be a superior bleaching method than the 0.25% potassium permanganate oxalic acid for three major reasons. Firstly the 10% H_2O_2 is a simple dilution to prepare reducing the risk of technical error across different users. Secondly the optimised protocol was effective, reproducible, and applicable to various tissue types with minimal section detachment. And thirdly the nuclear fast red counterstain enhanced the ability of viewing the bleached sections with greater ease in comparison to the neutral red counterstain used in the traditional Masson Fontana protocol.

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Telehealth in the management of diabetes in regional and remote First Nations populations in Australia: where are the gaps?

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Abstract

First Nations Australians in regional areas diagnosed with diabetes can face challenges such as difficulty accessing health care, leading to poor health outcomes. This study aimed to review the literature on the use of telehealth to manage diabetes in these populations.

A systematic review of the literature based upon PRISMA guidelines was conducted from 2010 to 2021 using keywords relevant to the management of diabetes via telehealth, with a focus on First Nations communities. While certain studies provide evidence of the role of telehealth in improving management of diabetes, there are certain disadvantages to telehealth such as patients not accessing vital pathology services and this can be detrimental to the long-term health of diabetic patients. Based upon the findings of this review, it is clear that more studies are required to investigate how telehealth could be used to improve health outcomes for First Nations populations with diabetes, not just in Australia but globally.

Keywords: ***telehealth, diabetes, First Nations, telemedicine, chronic illness***

Introduction

Diabetes is a serious chronic disease, which if poorly managed, may lead to complications such as blindness, renal failure, cardiovascular complications, strokes and amputations (Al-Badri and Hamady 2021).

Telehealth offers an alternative to face to face consultations for patients in regional and remote areas managing diabetes by creating an engaging platform for patients to achieve successful self-management of their condition to prevent any diabetes related complications (Al-Badri and Hamady 2021). Through the telehealth platform, patients typically spend more time examining electronic health records (EHR) than in a face-to-face consultation, promoting better patient engagement with their health care (Gu *et al* 2019).

First Nation Australians, many of whom live in remote and regional parts of the country can benefit greatly from accessing telehealth to improve the quality of care and management of chronic health conditions including diabetes (Hayman *et al* 2014; Moffatt and Eley 2010). This is particularly important as First Nations populations are identified as being more at risk of getting Type 2 diabetes (T2D) and related complications (Zimmet *e t al* 2016). Studies currently examining the role of telehealth, which also includes telemedicine, in health care delivery for First Nations in Australia are limited. This study aimed to review the literature relating to diabetes care via telehealth in remote and regional First Nation populations in Australia and globally.

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Method

Review Protocol

This review was conducted following PRISMA guidelines (Moher *et al* 2008). The following five databases were searched: SCOPUS, PubMed, Canadian Journal of Diabetes Database (CJD), Cochrane library and Cochrane Database of Systematic reviews (BMC). The Scopus database was used to find journals relevant to technology and health, filtered under health professions and health information management. PubMed search was used for its huge database on health-related science, CJD was chosen for its extensive resources on diabetes, Cochrane library for a range of resources on healthcare and BMC was chosen for its search on systematic reviews.

Several keywords including “Indigenous”, “First Nations” and “chronic illness” were used in combination with “telehealth” and “telemedicine”, resulting in 6191 papers (Table 1). These articles were then screened as described below.

***Table 1**. Keywords used in database searches in the initial searching for articles.*

Keywords (number of articles)	Database	References
"telehealth and diabetes" (2, 749) **"telehealth and first nations"** (46) **"telehealth and chronic illness"** (375) **"telemedicine and first nations"** (42) **"telemedicine and diabetes"** (2,580)	PubMed	5792
"telehealth and diabetes" (25) **"telehealth and first nations"** (7) **"telehealth and chronic illness"** (8)	Canadian Journal of Diabetes (CJD)	40
"telehealth and diabetes" (231) **"telehealth and first nations"** (35) **"telehealth and chronic illness"** (65)	Cochrane Library	331
"telemedicine and diabetes"; **"telemedicine and first nations"**; **"telemedicine and chronic illness"** (8) - filtered under health professions and health information management	SCOPUS	8
"telehealth and diabetes" (8) **"telemedicine and first nations"** (5) **"telehealth and chronic illness"** (7)	Cochrane Database of Systematic reviews (BMC)	20

The position paper’s requirements were either objects (nouns) or actions (verbs) for implementation purposes. Relevant objects and actions were tagged and numbered.

Inclusion criteria

Articles published in English in peer-reviewed journals between 2010-2021 were used in this review. Studies measuring HbA1c levels of Type 1 diabetes (T1D) or/ and T2D through telehealth/telemedicine or m-health/ e-health or technology, studies evaluating economic cost/travel time using telehealth, studies measuring telehealth effectiveness and studies evaluating patient outcomes/satisfaction using telehealth were included. Once duplicate articles, those not published in English and those that did not cover First Nations, diabetes and telehealth or telemedicine were excluded, a total of 77 papers were identified. Some of these papers were then further excluded as they were review articles and did not provide primary data. A total of 49 papers were finally included in the review.

Results and Discussion

Telehealth has become a key focus in improving healthcare in both remote and regional areas. When selecting papers for this review, it was evident that there is relatively little data that is specifically focussed on telehealth in the management of diabetes in First Nations populations and not just in Australia. This review therefore looked at telehealth more broadly relating to the management of diabetes overall in rural and remote populations.

Globally the use of telehealth in both developed and developing countries has increased over time with the implementation of mobile clinics and advancement in smartphone technology (Wilson and Maeder 2015; Ashrafzadeh *et al* 2019; Baptista *et al* 2016). The integration of personal health records in telehealth can aid in identifying patients requiring support can alert physicians regarding immediate care, and can alert patients to health resources (Sabo *et al* 2021; Gustafson *et al* 2021). There is unfortunately socio-economic disadvantage among remote/regional populations accessing telehealth services. Necessary actions are required to remedy this including the recruitment of First Nations people into the health workforce to ensure patients with chronic illness can access culturally appropriate health care via telehealth. Older First Nations diabetic patients need to be familiar and well adapted to changing technology. As some remote areas lack technological resources, implementation of technology in those areas should recognise the needs of the elderly, as well as to adapt technology to the culturally specific ways of the community. This inclusion of culture in technology can create a meaningful and culturally safe way of delivering telehealth (Jones *et al* 2017).

Diabetes management

The cost of patient travel to access health services in remote areas presents an economic burden for both patients and health professionals. (Carroll *et al* 2011; Snoswell *et al* 2019). With telehealth services patient travel would be reduced and access to healthcare would increase, regardless of location (Snoswell *et al* 2019; Thomas *et al* 2014; Nölke *et al* 2015). A cost-analysis by Thomas *et al* (2014) was undertaken of primary diabetes care in the Northern Territory in remote areas measuring hospitalisation, avoidable hospitalisation and mortality. Patients who used primary care 2-11 times per year, had lower rates of hospitalisation. Ensuring access to primary care in remote areas leads to health benefits and cost savings. Thaker *et al* (2013) examined the impact of telehealth on cost saving in Northern Queensland, it was found that telehealth reduced costs due to less travel expenses particularly for specialists. Estimated savings from reduced travel for patients and health care providers over the period of the study (56 months) were over AUD $762,000. The authors of the study proposed that these savings could be used to enhance other rural health facilities and services.

Patient experience with telehealth has generally been positive (Mooi *e t al* 2012). For example, a study by Roberts *et al* (2015) showed that First Nations patient satisfaction with telemedicine was positive. In this study high patient satisfaction levels were reported for both First Nation and non-First Nation participants, reaffirming the positive impact of telehealth in remote settings, with a higher preference for telehealth rather than standard face-to-face care. Similar findings have been reported by others (Caffery *et al* 2017; Sequist *et al* 2011). Some data is not recent, therefore further studies in the Australian context are urgently required. Importantly, from both healthcare and patient perspectives, telehealth has proven beneficial to breaking healthcare barriers allowing patients to access health services from their own homes and enhancing their ability to interact with multidisciplinary health care teams that may include nurses, dieticians and other health care providers who usually would not be readily accessible in a rural location (Kobe *et al* 2020; Nadar *et al* 2019; Al-Badri and Hamady 2021).

Part of diabetes management requires control of blood glycosylated haemoglobin A1C (HbA1c) levels to ensure optimal levels to prevent the risk of complications. Technological advancements have led to availability of devices to monitor and upload self-monitored blood glucose (SMBG) data to secure cloud-based databases. Barker *et al* (2016) investigated the impact of telehealth in diabetic care and management in Appalachia, a rural area in the USA, identifying the inability of access to diabetes education as a key concern for rural patients, a gap telehealth could fill. Similarly, others have reported on remote patient monitoring leading to reductions in HbA1c levels (Kirkland *et al* 2021; Tildesley *et al* 2015). The use of internet blood glucose monitoring systems (IBGMS) allowed for patients to efficiently manage their diabetes without the risk of hypoglycaemia, whilst preventing the risk of cardiovascular complications and increasing the quality of life. The increased patient response and self-motivation and the improved patient-clinician communication is a factor in the success of telehealth and the safety and effectiveness of IBGMS is recommended for patients (De Groot *e t al* 2021; Amante *et al* 2021). Due to patient engagement with their treatment through telehealth, remote patients can receive education and provide feedback to their caregivers which creates a better understanding of their condition and prioritise goals (Bergenstal *et al* 2021; Piette

et al 2016; Amante *et al* 2021). Telehealth services can also help with monitoring patients for complications of diabetes such as diabetic retinopathy. This would usually require support from a local health centre to provide access to a portable camera for retinal photographs in a setting where access to an ophthalmologist may not be possible (Gadkari, 2018; Mansberger *et al* 2013; Kim and Driver 2015).

Self-monitoring alone may not be adequate and a major gap in telehealth currently is the lack of support for patients in accessing pathology services for laboratory tests, which are required for patients' long term health (Hardie *et al* 2022).

While efficacy and safety of telehealth in helping with glycaemic control has been evident in many studies, patient clinical needs such as Quality of Life (QoL) have not been successfully investigated. Future studies on diabetes management should include family members and feedback from patients to gain a better understanding of how the QoL of patients could be enhanced (Amante *et al* 2021).

Despite the clear benefits of telehealth for patients in remote and regional areas, some researchers do not deem telehealth to be appropriate as a replacement of standard face-to-face care as clinical services such as pathology services and high risk patient monitoring for diabetes complications may not be easily undertaken online (Gaudillère *et al* 2021). Furthermore, some studies show that the benefits of telehealth in the management of diabetes may be unclear and require further investigation (Faraque *et al* 2017; De Groot *et al* 2021).

Diabetes care via telehealth during COVID-19

Recently the importance of telehealth has been recognised due to the COVID-19 pandemic. The pandemic has been an opportunity to evaluate the benefits of telehealth more broadly across the population in many countries. A study in Singapore by Lim *et al* (2020) conducted on adolescents diagnosed with T1D or T2D who require insulin therapy or oral hypoglycaemic agents showed that although patients still had to attend a clinic for blood tests and other services that cannot be provided online, having ongoing consultations via telehealth resulted in high patient satisfaction (Lim *et al* 2020). This study also showed that telehealth was considered similar to face-to-face consultations and some study participants deemed it to be superior. In addition, it was found that some patients would continue to use telemedicine where possible as an alternative to face-to-face consultations with a health care provider. Similar findings have been reported demonstrating the benefits of telehealth in managing diabetes in a pandemic (Goh *et al* 2021; Alharthi *et al* 2021). Overall, telehealth has been a critical component in delivering care during the COVID-19 pandemic (Agarwal *et al* 2021).

Overcoming barriers to telehealth in remote communities

A primary goal with the adoption of telehealth is to increase access for rural and remote communities and socio-economically disadvantaged areas. Studies reviewed have explored the importance and benefits of telehealth, and the need for its implementation globally to promote health literacy and improve health outcomes for the population. Discussion of the opportunities presented by telehealth was further examined by Seto *et al* (2019) on telemedicine in Yukon, identifying sustainability, quality improvement, and scalability as factors to consider in the expansion of telehealth in remote areas. The study concluded that based upon clinician and patient experience, the Yukon Telehealth System was found to be underutilised. Possible reasons could be; overworked and limited staff and maximum number of patients, or lack of interest by physicians and healthcare providers. The authors identified these factors as needing to be addressed to expand telehealth in remote areas.

There is an important role for telehealth is enhancing health literacy. A study by Ghaddar *et al* (2020) on e-health in underserved Hispanic communities demonstrated the importance of positive attitudes in e-health literacy with a positive attitude towards telehealth leading to enhanced e-health literacy and patient acceptance and use of telehealth.

Research also shows higher uptake of telehealth by younger adults and lower uptake by older patients which is concerning as older patients would be at higher risk of diabetic complications (Beck *et al* 2014; Thackeray *et al* 2013; Crico *et al* 2018). While these studies did not focus on First Nations populations it is likely that these findings are relevant to these populations in rural and remote areas.

There are a series of challenges associated with telehealth (Mahar *et al* 2018) including variable access to broadband and security breaches (Al-Badri and Hamady 2021). For these reasons telehealth-based solutions require experienced staff and relevant technical support (Aberer *et al* 2021). Where access to technology is an issue, use of telephone consultations can also lead to good outcomes and this is an important consideration for patients in remote and regional locations (Barker *et al* 2016; Nundy *et al* 2014; Al-Badri and Hamady 2021; Chin-Jung *et al* 2021; Kim *et al* 2016; Heintzman 2015).

In order for telehealth to be successfully implemented in the management of diabetes in regional and remote

First Nations populations, mobile clinics would be important (Dawson *et al* 2021). Through mobile diabetes clinics, nurses and laboratory personnel can provide point-of-care testing, which is an essential part of diabetes management.

Conclusion

There have been multiple studies in favour of telehealth as an alternative solution for overcoming barriers in the management of diabetes. More studies looking at the use of telehealth in the management of diabetes in First Nation populations in Australia are required. A significant gap is how patients accessing telehealth services could be better supported in accessing services that require them to physically attend a clinic for tests such as blood tests, X-rays and scans which are critical for their long-term health. Future studies should include an investigation of how mobile pathology services could support and be included as a key component of telehealth services to support diabetic patients in these communities.

Conflict of Interest

The authors have no conflicts of interests to declare.

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Juvenile myelomonocytic leukaemia case study

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Abstract

Juvenile myelomonocytic leukaemia (JMML) is a myeloproliferative disease that occurs in childhood and is characterised by the excessive production of myelomonocytic cells. According to the World Health Organization (WHO), JMML is classified in the overlapping myelodysplastic syndrome (MDS)/myeloproliferative neoplasm category. It also shares some clinical and molecular characteristics with chronic myelomonocytic leukaemia, which is a similar disease in adults. The majority of JMML cases occur between birth and the age of six, with a median age of two. Given its rarity and similar clinical findings with other myeloproliferative neoplasms, JMML is challenging to diagnose. For long-term survival most patients with JMML need allogeneic stem cell transplants. It has been observed however that many children with Casitas B-lineage lymphoma (CBL) mutations and neuroblastoma rat sarcoma (NRAS) mutations will experience spontaneous disease regression. This case report describes a case JMML with a CBL mutation in a nine-month-old boy with a unique clinical and laboratory presentation.

Keywords: ***Juvenile myelomonocytic leukaemia (JMML), myelodysplastic, myeloproliferative, allogenic stem cell transplant, mutation***

Introduction

Juvenile myelomonocytic leukaemia (JMML) is a rare but aggressive clonal haematopoietic disorder in childhood (Loh 2011; Sakashita *et al* 2016; Arora *et al* 2020). It is characterised by an overproduction of myelomonocytic cells and infiltration of leukaemic cells in multiple organs (Chang *et al* 2014; Sakashita *et al* 2016). It accounts for less than 2–3% of all leukaemias and has an estimated incidence of 1.2 cases per million persons per year (Arora *et al* 2020). It affects children with a median age of two years, and males more frequently than females by approximately 2:1.

The common clinical manifestations include fever, pallor, lymphadenopathy, hepatosplenomegaly, leuocytosis, absolute monocytosis, anaemia, and thrombocytopenia (Chang *et al* 2014; Sakashita *et al* 2016; Arora *et al* 2020). JMML shares some similar clinical findings with other diseases including chronic myelomonocytic leukaemia, Wiskott-Aldrich syndrome and certain viral infections, resulting in difficult diagnosis (Sakashita *et al* 2016). The WHO requires that the diagnosis of JMML fulfils the criteria from Category 1 and at least one of the criteria from Category 2 or meets at least two criteria from Category 3 (Table 1). Some characteristic features include monocytosis in peripheral blood (PB), less than 20% blasts in PB and bone marrow (BM), an absence of the BCR-ABL1 fusion gene, and increased haemoglobin F (HbF) (Arber *et al* 2016). Almost 90% of patients with JMML have somatic or germline mutations in genes associated with the RAS/MAPK (mitogen-activated protein kinase) signalling pathway, including PTPN11, NF1, NRAS, KRAS, and CBL (Chang *et al* 2014; Devorak *et al* 2014; Niemeyer *et al* 2014; Ganapathi *et al* 2015). These mutations define genetically and clinically distinct JMML subtypes and are becoming increasingly important for diagnosis and clinical decision making (Loh 2011; Gupta *et al* 2021).

JMML has a variable clinical course (Emanuel 2008; Sakashita *et al* 2016). Approximately one-third of patients experience rapid disease progression, while two-thirds experience relatively indolent progression (Sakashita *e t al* 2016). As of now, there is no proven chemotherapy for achieving complete remission in JMML; only allogeneic haematopoietic stem cell transplantation (HSCT) is proven to be a curative option for children with this malignancy (de Vries *et al* 2010; Niemeyer *et al* 2014; Locatelli *et al* 2015; Sakashita *et al* 2016; Gupta *et al* 2021). Some researchers have shown that

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some genetic variants of JMML may respond well to no or minimal chemotherapy although the majority will require a haemopoietic stem cell transplant (HSCT) to achieve a cure (Chang *et al* 2014; Locatelli *et al* 2015; Sakashita *et al* 2016; Su *et al* 2020). This case report highlights a rare clinical and laboratory presentation of JMML with Casitas B-lineage lymphoma (CBL) mutation in a nine-month-old boy.

***Table 1**. WHO 2016 criteria for the diagnosis of JMML (Adapted from Arber et al, 2016).*

Category 1 (All are required)	**Category 2** (One is sufficient)	**Category 3** (Patients without genetic features must have the following in addition to category 1)
Clinical and haematological features	**Genetic studies**	**Other features**
• Splenomegaly • Peripheral blood monocyte count > 1x 10^9/L • <20% blasts in the peripheral blood and bone marrow • Absence of the *BCR-ABL1* fusion gene	• Somatic mutation in *KRAS*, *NRAS* or *PTPN1* • Clinical diagnosis of *NF1* or *NF1* mutation • Germline CBL mutation and loss of heterozygosity of *CBL*	Monosomy 7 or other chromosomal abnormality, or at least 2 of the criteria listed below: 1. Circulating myeloid or erythroid precursors 2. Increased haemoglobin F for age 3. *GM-CSF* hypersensitivity

***NF1**: neurofibromatosis type 1; **CBL**: Casitas B-lineage lymphoma; **PTPN**: protein tyrosine phosphatase non-receptor type; **KRAS**: Kirsten rat sarcoma; **NRAS**: neuroblastoma rat sarcoma; **GM-CSF**: granulocyte-macrophage colony-stimulating factor.*

Case report

A nine-month-old boy with fever and hepatosplenomegaly was admitted to the Emergency Department. A full blood count result revealed that the white blood cell count was 73.50 x 10^9/L (RR for age: 3.5–11.0 x 10^9/L), haemoglobin was 62 g/L (RR for age: 104–132 g/L), platelet count was 36 x 10^9/L (RR for age: 205–553 x 10^9/L), monocyte count was 14.55 x 10^9/L (RR for age: 0.2–1.1 x 10^9/L), and 18 nucleated red blood cells (NRBCs)/100 WBC. An examination of a blood film showed a leukocytosis with a marked left shift, monocytosis, 2% of dysplastic blasts, and large and occasionally hypogranular platelets (Figures 1 and 2).

Bone marrow examination showed marked hypercellularity with varying degrees of myeloid hyperplasia and approximately 6% blasts. The blasts were large and resembled myeloblasts and had a high nuclear to cytoplasmic (N/C) ratio, basophilic cytoplasm, fine azurophilic granules and prominent nucleoli. Erythropoiesis was relatively preserved in cellularity but reduced megakaryopoiesis and mild megakaryocytic dysplasia were present (Figure 3).

Flow cytometry analysis demonstrated positive expression of CD45, HLA-DR, CD13, CD33, CD34, CD38, and CD117. Fluorescence in situ hybridisation (FISH) showed a normal signal pattern. Cytogenetic analysis of bone marrow cells revealed a normal male karyotype (46, XY [20]) with no abnormalities. Coagulation tests were unremarkable, but serum lactate dehydrogenase (LDH) was increased to 516 U/L (RR <250U/L). HbF level was markedly elevated, at 17.0% (RR for age: 1.5–3.5%).

The laboratory results of this patient fulfilled the diagnostic criteria for Categories 1 and 2 and some of the criteria in Category 3 including circulating myeloid or erythroid precursors and increased HbF for age (Table 1). As a result, the patient was classified as JMML. A molecular genetic test was conducted as part of the clinical risk assessment and evaluation of treatment indicated that the patient has a germline CBL mutation. The initial diagnosis was two years ago and the patient remains highly transfusion dependent but without chemotherapy or an HSCT.

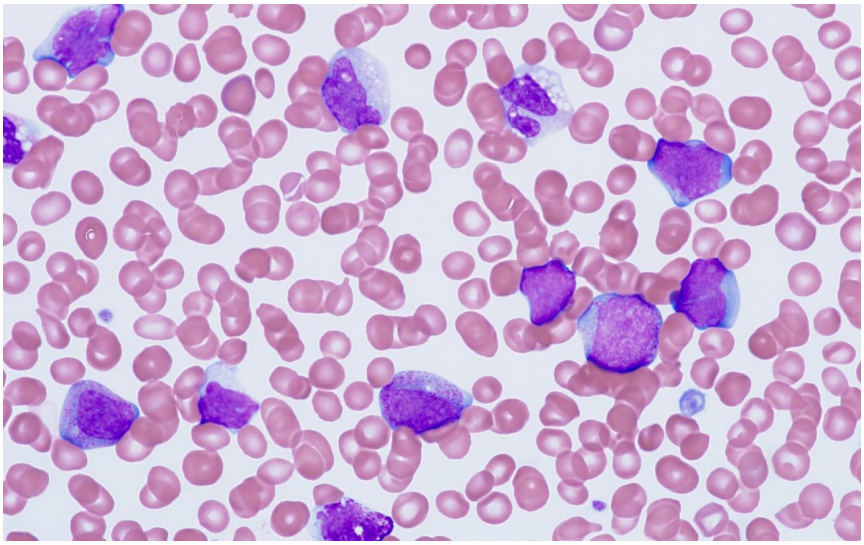

Figure 1. *Peripheral blood film showing an increased number of myeloblasts and mature monocytes.*

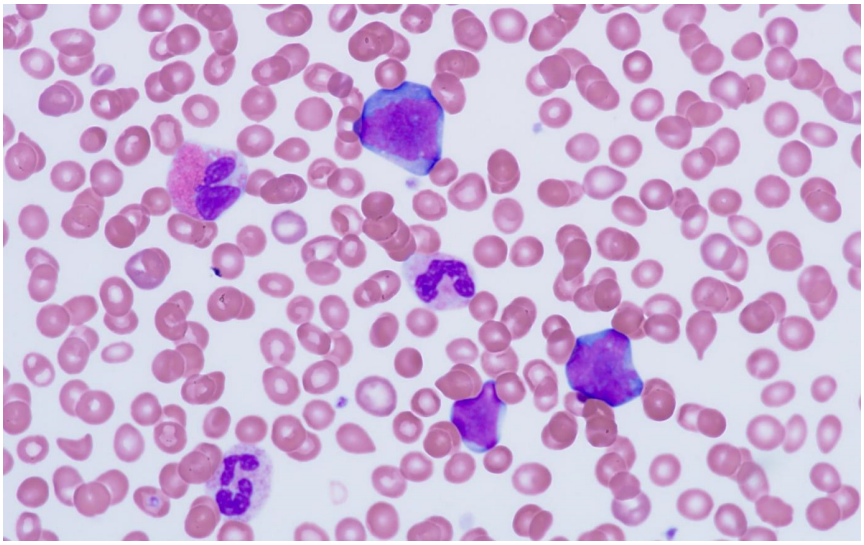

Figure 2. *Peripheral blood film showing myeloblasts, neutrophils and eosinophils.*

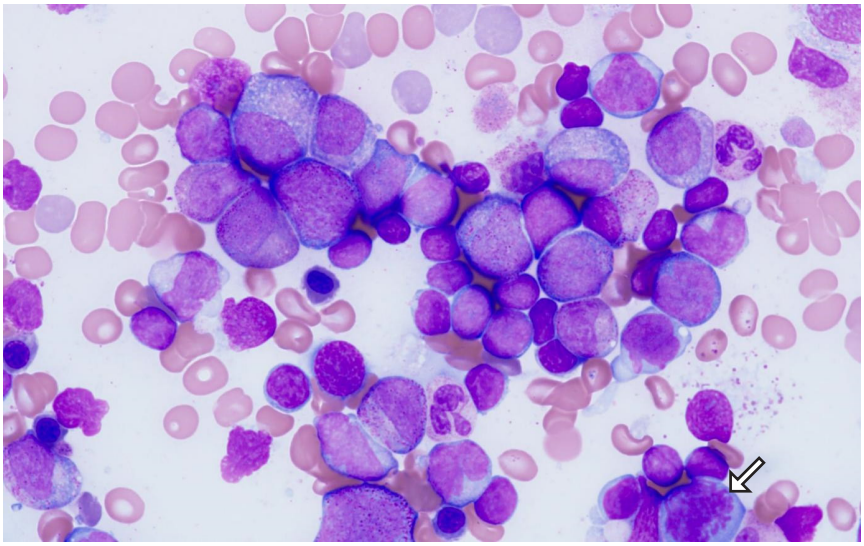

Figure 3. *Bone marrow aspirate showing an increased number of myeloid precursors, myeloblasts and a mitotic figure (shown by the arrow).*

Discussion

JMML is an aggressive myeloid neoplasm that occurs in childhood and is characterised by overproduction of myeloid progenitors and infiltration of malignant cells in multiple organs (Loh 2011; Chang *et al* 2014; Arora *et al* 2020; Gupta *et al* 2021). JMML is most often found between the ages of one month and six years, with a median age of two years at presentation (Sakashita *et al* 2016; Arora *et al* 2020; Gupta *et al* 2021). In most cases, a patient will present with non-constitutional symptoms or symptoms associated with an early infection. Hepatosplenomegaly (90%) is usually present, as seen in this case, as well as non-haematopoietic tissues that are affected by leukaemic infiltration (Loh 2011; Chang *et al* 2014; Sakashita *et al* 2016; Arora *et al* 2020; Gupta *et al* 2021).

JMML is diagnosed according to several standards established by the WHO. Clinical findings and laboratory investigations play a key role in diagnosis, including peripheral blood film, flow cytometry, bone marrow examination and genetic studies. Most cases show a striking monocytosis with immature monocytes and myeloblasts in peripheral blood film (Loh 2011; Arber *et al* 2016; Sakashita *et al* 2016). Bone marrow examination must identify fewer than 20% myeloblasts, show abnormal myelopoiesis or megakaryopoiesis in varying degrees, and no evidence of BCR/ABL-1 translocations (Chan *et al* 2008; Loh 2011; Arber *et al* 2016; Sakashita *et al* 2016). Around 65% of children with JMML have a normal karyotype whereas approximately 25% of patients have monosomy 7 and other abnormalities occur in another 10% of patients (Locatelli *et al* 2015; Niemeyer 2018). Approximate 50% of patients with JMML have elevated HbF due to an altered haemoglobin pattern and show hypersensitivity of haemopoietic progenitors to granulocyte-macrophage colony-stimulating factor (GM-CSF) (Chan *et al* 2008; Loh 2011).

In recent years major advancements have been made in defining the genomic landscape of JMML (Chang *et al* 2014; Dvorak *et al* 2014). The identification of contributing genetic mutations in PTPN11, NF1, NRAS, KRAS, and CBL that encode RAS/MAPK pathway signalling proteins has greatly enhanced our understanding of the pathogenesis of JMML (Loh 2011; Chang *et al* 2014). Mutations in this pathway are frequently found to have a mutually exclusive effect resulting in hyperactivation and uncontrolled proliferation of cells (Chang *et al* 2014). This discovery has greatly contributed to the definitive diagnosis of JMML patients and provides better clinical outcome prediction as well as treatment strategy (Loh 2011; Chang *et al* 2014).

JMML has a poor prognosis with only approximately 50% of patients surviving after an allogeneic stem cell transplant (Gupta *et al* 2021). The median survival time of patients without HSCT can be as short as 10–12 months because of monocyte infiltration in non-haemopoietic organs, bleeding, infection and organ failure (Locatelli *et al* 2015). There are several factors associated with poor prognosis including older age (≥2 years), a low platelet count at diagnosis (< 33 x 10^9/L) and high HbF levels (>10%) (Locatelli *et al* 2015; Niemeyer 2018).

According to Gupta *et al* (2021), there are a diverse range of chemotherapy protocols are used to treat JMML such as the intensive protocols used in acute myeloid leukaemia (AML) therapy, milder leukaemia maintenance schemes, and single-agent chemotherapy. HSCT is currently considered as a curative therapy for patients with JMML and can cure about half of them (Locatelli *et al* 2015). Interestingly some patients with somatic NRAS mutation or germline mutation in CBL have shown improvement in their haematological condition with minimal or no treatment (Locatelli *et al* 2015). An international study had shown that four of six JMML patients with CBL mutations who did not receive HSCT survived without evidence of JMML for 7.5 to18 years after initial diagnosis (Locatelli *et al* 2015).

In conclusion, the patient described in this case study was diagnosed with JMML with CBL mutation two years ago with subsequent frequent hospitalisation for respiratory infection and anaemia but with no treatment intervention such as chemotherapy or HSCT so far. The patient remains highly transfusion dependent. Children with JMML with CBL mutation may only require continuous medical monitoring but with necessary treatment refinement should there be a disease progression.

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Questions relating to the article *'A validation of at-home infant stool sample collection devices for determining the faecal microbiome'* at page 2 of this issue.

1.	The modulation of bacterial population in the gut could provide long term metabolic and immune benefits.	True/False
2.	A volume of 150 µl was chosen for the initial sample volume for the study.	True/False
3.	There was a difference in results with samples stored using the OMNIgene® GUT kit to the same samples collected in a standard/sterile collection tube and stored at - 80°C for 3 months prior to testing.	True/False
4.	Samples in DNA/RNA Shield and RNA later preservation buffers have to be stored refrigerated or frozen to preserve genetic integrity.	True/False
5.	Inadequate storage protocols can lead to DNA/RNA fragmentation in less than 4 hours at room temperature.	True/False
6.	Faecal samples stored on ice for up to 48 or 4°C for 24 hours is sufficient if samples are to be processed immediately.	True/False
7.	A dysbiotic microbiome puts preterm infants at a greater risk of necrotising enterocolitis (NEC) and sepsis.	True/False
8.	The adequacy of the DNA extract can be assessed by estimating the read depth captured during sequencing.	True/False
9.	There is no link between an immature gut microbiome to chronic diseases, such as asthma and diabetes.	True/False
10.	Probiotic supplementation has been shown to be of benefit in preterm infants.	True/False

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Questions relating to the article '*Dilute hydrogen peroxide: an alternative bleaching method for the Masson Fontana Silver stain*' at page 11 of this issue.

1.	Eumelanins are the most common type of melanin found in human tissue and consist primarily of 5,6 dihydroxyindole units.	True/False
2.	Melanins are low molecular weight complex pigments.	True/False
3.	The KIT, BRAF and NRAS mutations have been found in a percentage of melanoma cases.	True/False
4.	Melanin is susceptible to bleaching with strong oxidants such as potassium permanganate and hydrogen peroxide.	True/False
5.	Melanin, a brown-black pigment, is only found in animals.	True/False
6.	The physical masking effects of melanin pigment can impact the histological and molecular diagnosis of melanocytic lesions.	True/False
7.	The Masson Fontana is a silver staining technique widely used for the demonstration, localisation, and quantification of melanin in skin pigmentation and disease.	True/False
8.	Pheomelanins produce yellow and red coloured pigment.	True/False
9.	Melanin pigment has the capacity to absorb a wide range of ultraviolet radiation, but this does not interfere with the accurate photometric quantification of nucleic acids.	True/False
10.	The optimised hydrogen peroxide protocol proved to be a superior bleaching method than the 0.25% potassium permanganate oxalic acid.	True/False

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Therefore, the extended time frame due to Covid-19 will no longer apply.

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Immunohistochemistry: a technical guide to current practices

Trung Nguyen (Ed)

Cambridge University Press 2022, 282 pages, paperback

Paperback 282 pages

RRP: $141.95

ISBN: 9781009107723

This book is an excellent technical guide for all medical laboratory scientists and technicians performing immunohistochemistry (IHC).

In the first part of the book, the authors give the reader an in-depth understanding of IHC fundamentals by taking the reader into its history, development and basic principles. Chapter 1 serves as an introduction to IHC principles and the people credited for its discovery and development. It is such an important tool in research applications and diagnostic endeavours for all clinicians, scientists, laboratory staff and managers to understand its fundamentals before exploring its wonders.

It is well-written and principles are described along with illustrations, helping the readers understand the bigger picture without being overwhelmed. The development of antigen retrieval methods and polymer detection systems is also discussed by the authors. This included the various labels acquired, and the process involved to detect them. Likewise, technologies invented to increase sensitivity and standardization efforts are raised. The authors also guide readers through a good quality management system and cited a very helpful template. This chapter not only serves as a reminder of the importance of "quality" matters in IHC, but also allows an appreciation of the huge hurdles that must be overcome to guarantee a reliable and accurate IHC stain.

The guide covers the three main automated platforms that are currently used in performing IHC. Firstly, the Leica Bond III, a reliable, efficient and fully automated instrument with robotics for immunohistochemistry. The principles of the Leica Bond III instrument are explained thoroughly as well as its polymer detection kits and the bond staining protocols. Bond quality control and maintenance of the bond are well-written to guide the users.

Secondly is the Roche Ventana Benchmark Ultra. The unique technology behind Ultra lies in the Ultra LCS, individual slide drawers and ability to perform manual primary antibody application. The authors also explained the machine's principles well.

And the last machine that the authors sighted in this guide is the Agilent Dako Omnis, although a large instrument, its modern design aesthetics with attached touch screen and status light bar are the highlights. There are great explanation of its principles and robust quality control systems.

Sufficient details are provided on how technology has been applied to automating the process of IHC. Each of the machines covered are well labelled and principles are explained in a very concise manner. Each commercially available, automated IHC platform has strategic design differences with their own advantages and disadvantages. Understanding these differences can help match the demands of testing volumes, turnaround times, standardization and labour savings toward the appropriate instrumentation.

In research applications, there is no limit when it comes to IHC, and the authors direct the readers to a strategic approach and technical tips. The chapter on troubleshooting served as a treatise on why things can go awry and offered practical advice to readers on how to rectify problems encountered in IHC.

The final chapter discusses the current status of IHC and the future of IHC applications, which is very exciting for the readers. As I read, it takes me to what I am actually doing on the bench, what is happening and how I can make improvements.

Comprehensive yet practical and concise, the Immunohistochemistry, technical guide to current practice will be of great value for medical laboratory scientists, technicians, managers, pathologists and clinicians alike.

Reprinted book review by Rocel Mangaliman. Immunohistochemistry: a technical guide to current practices. NZJMLS 2022; 76(3): 163.

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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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Examples of the correct form for references are given below:

Journal Reference:

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 USB stick. 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.

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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