The investigation of bronchoalveolar lavage fluid in paediatric chronic wheezers.

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Declaration

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Abstract

Background: Paediatric broncho-alveolar lavage (BAL) is a minimally invasive procedure, performed during flexible bronchoscopy using isotonic saline to obtain a sample of bronchial and alveolar fluid from the distal lung. The BAL sample provides a determination of the different cell types that are present in the alveolar space as well as establishing the presence of lipid or haemosiderin in alveolar macrophages. In paediatric clinical practice, the patients that undergo BAL most frequently are children with chronic wheezing and recurrent chest infections as part of their workup to determine the possible cause. However, the interpretation of these results, in the clinical context is relatively uncertain. We therefore conducted this study in collaboration with the paediatric pulmonology unit at Tygerberg hospital in an attempt to determine if the BAL specimens can be used to categorize children with wheezing into either a gastro-eosophageal reflux or primary respiratory pathology group. We wanted to guage whether laboratory tests such as the differential cells counts; lipid-laden macrophage index, haemosiderin-laden macrophages index and number of carbon-laden macrophages can be used as a marker for pulmonary pathology. To determine if BAL can assist with sub-classification of the primary respiratory pathology group and determine if the amount of carbon-laden macrophages corresponds to the risk of increased lung disease

Aim: Henceforth, we undertook this study to determine if the BAL specimens could be used to categorize these children as belonging to either the gastro-esophageal reflux or primary respiratory pathology group by grading the amounts of Oil red O lipid-laden macrophages (LLM) which is a marker for gastro-oesophageal reflux aspiration , Perl's Prussian blue positive haemosiderin-laden macrophages(HLM) which serves to indicate the presence of pulmonary haemorrage and carbonladen macrophage (CLM) which may contribute to chronic lung disease, however has not yet been assessed in our setting. We also wanted to determine if the amount of phagocytosed carbon by alveolar macrophages had any correlation with chronic wheezing.

Methods: Between march 2017 and march 2018, 68 paediatric patients presenting with a chronic wheeze underwent clinical investigations which included routine chemical pathology, microbiology and virology testing and the cytological evaluation of their BAL specimens. Once processed, the slides were stained with the Papanicolaou, Giemsa, Oil red O and Perl's Prussian blue to quantitate the differential cell count as well as lipid-laden macrophage index, haemosiderin-laden macrophage index and number of carbon-laden macrophages present. .

After a full clinical work-up, these patients were placed into a broad category of recurrent wheeze and recurrent infection, and sub-category groups of gastro-eosaphageal reflux disease (GORD), alveolar proteinosis, idiopathic pulmonary haemosiderosis (IPH), recurrent wheeze, infection/inflammation and structural abnormalities.

Results: There was no statistical significance when comparing the cellular differential counts; carbon; lipid and haemosiderin-macrophages; tuberculosis investigations; cystic fibrosis investigations; virology investigations; microbiology investigations and c-reactive protein in the broad categories of recurrent wheezer vs. recurrent infection as well as subcategories of infection/inflammation, GORD, structural abnormality, idiopathic wheezer, IPF and alveolar proteinosis.

Conclusion: The cytological investigation of paediatric BAL specimens continues to play a role in the clinical work up of children with chronic wheezing. This study did not manage to yield statistically significant data to identify a specific underlying cause and further research is needed in this field.

Opsomming

Agtergrond: Pediatriese brongo-alveolêre spoeling (BAS) is 'n minimaal indringende prosedure wat uitgevoer word tydens buigsame brongoskopie met behulp van isotoniese soutoplossings om 'n monster van brongiale en alveolêre vog te verkry. Die BASmonster help met die bepaling van die oorheersende seltipe en die bepaling van die teenwoordigheid van lipied of hemosiderien in alveolêre makrofage. In die kliniese praktyk is die pasiënte wat BAS gereeld ondergaan, kinders met chroniese fluit en herhalende borskasinfeksies as deel van hul kliniese ondersoek om die moontlike oorsaak te bepaal. Die interpretasie van hierdie resultate in die kliniese konteks is egter betreklik onseker. Daarom het ons hierdie studie in saamwerking met die pediatriese pulmonologie-eenheid in die Tygerberg hopitaal uitgevoer in ‘n poging om vas te stel of die BAS monster kan gebruik word om kinders met ‘n chroniese fluit in ‘n gastro-oesofiese refluks of ‘n primêre repiratoriese patologiegroep te kategoriseer. Ons wou vasstel laboratoriumtoetse soos die differensiële seltelling olie-rooi O-lipiedbelaaide makrofage, pruisiese blou positiewe hemosiderien belaaide makrofage en koolstof belaaide makrofage kan gebruik word as ‘n merker vir pulmonale patologie. Om vas te stel of BAS kan help met die subklassifikasie van die primêre repiratoriese patologiegroep en bepal of die nommer van koolstof belaaide makrofage ooreenstem met die risko van verhoogtde longsiekte.

Doelwitte: Ons het hierdie studie onderneem om te bepaal of die BAS-monsters gebruik kan word om hierdie kinders te kategoriseer onder die gastro-esofageale refluks- of primêre respiratoriese patologiegroep. Daarvoor word die aantal olie-rooi O-lipiedbelaaide makrofage (LLM), Pruisiese blou positiewe hemosiderien belaaide makrofage (HLM) en koolstof belaaide makrofage (CLM) bepaal. Ons wou ook vasstel of die aantal gefagositeerde koolstof in alveolêre makrofage met chroniese fluit verband hou.

Metodes: Tussen 13-Mar-2017 en-12-Mrt-2018, het 68 pediatriese pasiënte wat chroniese fluitbors gehad het, kliniese ondersoeke ondergaan wat roetine chemiese patologie, mikrobiologie en virologie-toetse en die sitologiese evaluering van hul BAS-monsters insluit. Nadat die skyfies gemaak is, is hulle met papanicolaou, giemsa, olie-rooi O en Pruisiese blou van Perl gekleur om die differensiële seltelling asook indekse van olie-rooi O-lipiedbelaaide makrofage, pruisiese blou positiewe hemosiderien belaaide makrofage ,en koolstof belaaide makrofage te kwantifiseer. Die beoordeling van die spesiale kleurmetodes is uitgevoer. Na 'n volledige kliniese ondersoek, is hierdie pasiënte in 'n breë kategorie van herhalende fluit en

herhalende infeksie geplaas, en sub-kategorieë gastro-esofageale reflukssiekte alveolêre proteïnose, idiopatiese pulmonale hemosiderose (IPH), herhalende fluit, infeksie / ontsteking en strukturele abnormaliteite.

Resultate: Daar was geen statistiese beduidende resultate by die vergelyking van sellulêre differensiaalgetalle nie; koolstof; lipied- en hemosiderien-makrofage; tuberkulose ondersoeke; sistiese fibrose ondersoeke; virologiese ondersoeke; mikrobiologiese ondersoeke en c-reaktiewe proteïen in die breë kategorieë van herhalende fluitwaarde vs herhalende infeksie, sowel as subkategorieë van infeksie / inflammasie, gastro-esofageale reflukssiekte, strukturele abnormaliteite, idiopatiese fluit, IPF en alveolêre proteïnose.

Gevolgtrekking: Die sitologiese ondersoek van pediatriese BAS-monsters speel steeds 'n rol in die kliniese opbou van kinders met chroniese fluit. Hierdie studie het nie statisties geslaag om ‘n spesifieke onderliggende oorsaak te identifiseer nie en verdere navorsing op hierdie gebied is nodig.

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To my fellow colleagues who process and interpret bronchoalveolar lavage samples, the staff involved in retrieving slides, my manager as well as everyone else at Tygerberg cytology- Individual commitment to a group effort is what makes a team work, a company function efficiently and has the ability to impact society, no words can describe how thankful I am to belong to such a great team.

Dedications

I dedicate this dissertation to Jesus, the author, creator and one who directs my every step. I don't want to do this life without your gentle guidance.

In loving memory of my belated parents, Dawn and Anthony Marshall.

Mom and dad, I know that you guys are smiling down from Heaven.

To my loving family and friends, thank you for getting me through the tough times.

To my dog Mikayla, thanks for being my study buddy. Finally, to Dr. Chase, thank you for imparting your wisdom and knowledge over tea. May the fruits of your labour continue and help children all over South Africa.

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1.	AM	Alveolar Macrophage
2.	AO	Auramine O
3.	BAL	Bronchoalveolar lavage
4.	BALF	Bronchoalveolar lavage fluid
5.	CB	Carbon black
6.	CF	Cystic fibrosis
7.	CLM	Carbon-laden macrophage
8.	GORD	Gastro-oesophageal reflux disease
9.	HLM	Haemosiderin-laden macrophage
10.	HLMI	Haemosiderin-laden macrophage index
11.	GXP	GeneXpert
12.	ICC	Immunocytochemical stain(s)
13.	IgA	Immunoglobulin A
14.	IgG	Immunoglobulin G
15.	IgM	Immunoglobulin M
16.	IHC	Immunohistochemical stain(s)
17.	LLM	Lipid-laden macrophage
18.	LLMI	Lipid-laden macrophage index
19.	MTB	*Mycobacterium tuberculosis*

20.NHLS	National Health Laboratory Services
21.ORO	Oil Red O stain
22.PAP	Papanicolaou stain
23.PBB	Perl's Prussian blue stain
24.PCD	Primary ciliary dyskinesia
25.PCR	Polymerase chain reaction
26.PCT	Procalcitonin
27.PTB	Pulmonary tuberculosis
28.PM	Particulate matter
29.RT-PCR	Reverse transcriptase polymerase chain reaction
30.SOP	Standard operating procedure
31.UFP	Ultrafine particles

Glossary

Allergy	An excessive or inapt immune reaction to an environmental agent (antigen).
Amyloid	An insoluble extracellular material that when deposited, causes hardening, enlargement and malfunction of organs.
Antibody	An immunoglobulin specific for a certain antigen.
Antigen	A substance binding to a particular antibody or antigen receptor.
Atopy	The genetic predisposition to develop allergic conditions and hypersensitivity conditions especially to inhaled and food allergens.
Autosomal (gene)	A gene residing on one of the 22 chromosomes other than a sex (X or Y) chromosome.
Axoneme	A fibrillary bunch whip-like appendages or cilia that usually consist of 9 microtubule pairs arranged around one single central pair.
Basophil	A bone marrow derived white blood cell with uniform blue-black granules involved in the immune response.
Basophilic	A cell or its components which has an affinity for a basic dye such as haematoxylin. Often staining dark blue in colour.
Biomass fuel	Plant or animal derived matter used for energy production such as heat or electricity.
Bronchiectasis	Irreversible dilation of the bronchi.
Bronchiolitis	Inflammation of the bronchioles.

Cilia	Fine hair-like structures projecting from certain cells such as those seen in the respiratory tract which assist in sweeping particulate matter along the mucociliary escalator.
Cytocentrifuge	A low-speed, low-acceleration centrifuge used to separate and deposit cells suspended in a liquid without damaging them on a slide for microscopic examination.
Cyanophilic	A cell or its components which have an affinity for blue and green dyes such as polychromatic EA-50.
Dendritic cell	An antigen presenting cell acting as a messenger between the innate and adaptive immune system. Its primary role is to process antigens and present them to the cell surface receptors of T-lymphocytes.
Eosinophil	A bone marrow derived white blood cell involved in the immune response launched against foreign substances and parasites.
Eosinophilic	cytoplasmic components which take up eosin dye and as a result stain bright pink.
Extrinsic	Pertaining to a cause outside a structure in the body causing compression and/or obstruction, or a cause external to the body.
Ferritin	A protein on erythrocytes responsible for storing iron.
Ferrocyanide	A salt containing the anion $Fe(CN)_6^{4-}$ 2 used to produce a blue pigment.
Fixation:	Immobilizing preserving, visualizing and hardening want to observe blue pigment in the demonstration of iron.

Free radicals	Chemical radicals with highly reactive unpaired electrons in their outer shell composition.
Glycogen	A carbohydrate polysaccharide substance deposited in tissues which forms glucose on hydrolysis.
Glycoprotein	A type of protein molecule that has a carbohydrate attached to it.
Good-pasture's syndrome	An autoimmune disease that attacks the lungs and kidneys.
Granulocyte	A category of white blood cells characterized by cytoplasmic granules which are released during infections, allergic reactions and asthma. They are also known as polymorphonuclear cells. These include neutrophils, eosinophils and basophils.
Granuloma	A structure formed during inflammation found in numerous diseases. Aggregates of epithelioid macrophages form, often including multinucleated giant cells which are also derived from macrophages (histiocytes) when the immune system attempts to wall off substances which it perceives as foreign but is unable to eliminate it.
Haematamesis	The vomiting up of blood.
Haemoglobin	A red iron containing protein in erythrocytes responsible for transporting oxygen via the blood to tissue.
Haemosiderin	A yellowish-brown granular pigment formed by the haemoglobin degradation.

Hilum	A wedge shaped section on the central middle aspect of each lung where bronchi, arteries, veins and nerves enter and exit the lung.
Hypersensitivity pneumonitis	Also known as extrinsic allergic alveolitis is a type of interstitial lung disease characterized by a immune mediated hypersensitivity inflammatory reaction to inhaled organic substances.
Hypochromasia	Pale staining nuclear chromatin.
Idiopathic pulmonary fibrosis	A category of chronic lung disease characterized by scarring/fibrosis of the lung for an unknown reason.
Immunocytochemistry	A laboratory staining technique used to demonstrate proteins and peptides on cytology specimens.
Immunohistochemistry	A laboratory staining technique used to demonstrate antigens using various antibodies on histology specimens.
Lamellar body	Secretory organelles found in type II pneumocytes of the lung and keratinocytes in the skin.
Lipoprotein	A substance containing both protein and lipid that transports lipid in the blood.
Mucociliary escalator	The self-clearing mechanism of the respiratory system to remove inhaled particulate and trapped substances from the airways.
Mucostasis	Stopping the secretion of mucus.
Monoclonal antibody	An artificially produced antibody produced by a single clone of cells or cell line.
Opsonisation	A process whereby opsonins such as antibodies coat foreign material and micro-organisms rendering them more susceptible to phagocytosis.

Organizing pneumonia	Buds of granulation within the alveolar spaces which progress from fibrin exudate to loose collagen containing fibroblasts subsequently forming collagen plugs within the alveoli.
Pathogenicity	The ability of a micro-organism to cause disease based on its virulence (high/low).
Phagocytosis	Ingestion of a particle or micro-organism by a cell
Pneumonia	Inflammation of either one or both lungs as a result of a bacterial or viral infection in which the alveoli become filled with an inflammatory exudate.
Polyclonal antibody	A collection of immunoglobulins made up by antibodies secreted by various B cell lines within the body to react against a specific antigen.
Polycyclic hydrocarbon	Organic compounds consisting of only carbon and hydrogen.
Polymerase chain reaction	A laboratory technique used to make multiple copies of a strand of a specific DNA or RNA segment.
Primer (molecular biology)	The strands of DNA or RNA serving as the initial foundation in the replication process and is used in the demarcation of the template strand to be amplified. 2 primers are therefore “matched” to the segment strand of DNA/RNA.
Probe	A blunt ended surgical instrument used to examine a wound or section of the body.
Psychogenic	A condition manifesting from a psychological origin as opposed to a physical one.
Real time PCR	Also amplification is detected in “real time” by using a fluorescent reporter. The signal strength of the fluorescent reporter is directly proportional to the amount of amplified DNA molecules.

Reverse-transcriptase PCR	A variation of the PCR method whereby the presence and amount of RNA is detected.
Recessive (genetics)	A heritable characteristic expressed in offspring when inherited by both parents. Such as the gene for blue eyes. A recessive gene can be masked by a dominant gene as is seen in the dominant gene for brown eyes.
Reticuloendothelial system	The mononuclear phagocytic system in the body consisting of various cells with the primary function to remove foreign substances, dead and abnormal cells.
Sputolysin	A mucolytic agent capable of thick mucus.
Trypan blue	An azo dye used as a biological stain to colour live cells of the reticuloendothelial system such as macrophages with intact membranes due to its absorption.
Virulence	The ability of a micro-organism to cause disease. Pathogenicity is based on a micro-organism's virulence factors such as adhesins, anti-phagocytic factors and ability to produce toxins

Chapter 1: Literature review

1.1 A brief history of the bronchoalveolar lavage, environmental air pollution and the relation of ultrafine particles to lung disease development.

1.1.1 A brief history of the bronchoalveolar lavage technique.

Bronchoalveolar lavage (BAL) allows for the sampling of the peripheral lung. This is a minimally invasive procedure with a low morbidity and is performed under local anaesthesia in adults and general anesthesia in children. Specimens are obtained by wedging a sub-segmental bronchus with the bronchoscope and lavaging the area with warm isotonic saline. Numerous consecutive fluid instillations of 20-100ml aliquots are performed, flooding the distal airspace before re-aspiration. The aspirated fluid should contain very few bronchial cells. BAL fluid can assist in differentiating between inflammatory processes which are lymphocyte predominant from those which are macrophage or neutrophil predominant. In addition, the presence of haemosiderin-laden macrophages and lipid-laden macrophages may aid in the diagnosis of pulmonary haemosiderosis and lipid pneumonia respectively. [1,2]

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1 .1.2 Structure of the lung and cellular constituents.

The respiratory tract is divided into the upper and lower respiratory tract respectively. The upper respiratory tract consists of the nasal cavity, paranasal sinuses as well as the pharynx. The lower respiratory tract comprises of the larynx, trachea, bronchi, bronchioles and alveolar ducts and sacs.

The trachea bifurcates into the left and right main bronchi at the carina. These bronchi undergo further branching until they form bronchioles. The bronchial tree is lined by pseudostratified glandular epithelium consisting predominantly of ciliated columnar and mucin-producing goblet cells. Cilia play a pivotal role in ensuring that mucus-trapped inhaled particulate matter swept upwards via the mucociliary escalator. Terminal bronchioles further divide to form alveolar ducts, alveolar sacs and alveoli. Alveoli are lined by type I and II

pneumocytes, the latter are surfactant secreting cells with the former being modified squamous cells involved in gaseous exchange.[1,3]

1.1.3 Environmental air pollution, ultrafine particles and the link to lung injury.

The lung is well evolved to handle airborne dust particles that are inhaled and deposited. Inhaled particulate matter may be divided into fibronic or non-fibronic. Fibronic particles such as asbestos and quartz are evidently pathogenic to the lung; however, non-fibronic "nuisance type" particles such as carbon and titanium oxide have been previously viewed as non-toxic. [4,5]

There are 4 types of macrophages found in the lung. These are the alveolar; interstitial; intravascular macrophage and the dendritic cell, with the alveolar macrophage being the only macrophage that is exposed to air. It has been observed that carbon pigment, which is black in colour, is not formed in vivo, but inhaled and ingested by alveolar macrophages. These carbon laden macrophages have an affinity for lymphatic tissue with migration to regional hilar lymph nodes.[5]

The worldwide urban population is projected to rise to 6.5 billion by the year 2050.[6] Particulate matter (PM) in air pollution is a mixture of solid and liquid particles varying in number, size, surface area, chemical composition and origin. [7]PM is broadly classified according to aerodynamic diameters:

- $PM_{10:}$ Coarse particles with a median aerodynamic diameter of 10µm or less.
- $PM_{2.5}$: Coarse particles with a median aerodynamic diameter of 2.5µm or less.
- $UFP<_{0.1}$: Ultrafine particles with a median aerodynamic diameter of 0.2µm or less.[7]

Most particles acquired from the ambient atmosphere of urban settings range in size from 0.05-0.2µm, with a chemical composition consisting half of carbon and half of salts such as ammonium sulphate or ammonium nitrate. Polycyclic aromatic hydrocarbons bind with diesel engine exhaust emissions and accounts for approximately 90% of the total particle mass in urban areas and major cities such as London.[8] Inhaled ambient PM_{10} is more likely to be removed by mucociliary clearance. Previous studies utilizing radioactive particles have demonstrated that particles with a median diameter of 2.5µm undergo a total lung deposition of 83% compared to particles in the size range of PM_{10}. In addition, $PM_{2.5}$ has a longer

retention time when compared with PM_{10}.[8] This appears to be as a result of mass vs surface area of a particle. Albeit that the mass of a 0.1µm particle is smaller than that of a 1µm particle, the surface area of the 0. 1µm particle is tenfold greater than that of the 1µm particle. As the particle decreases in size, its surface area and capacity to carry toxic substances and free radicals increases. More free radicals have been found in UFP samples in comparison to coarser particles of the same substance. The consequences of PM and UFP exposure and their ability to carry toxic substances and free radicals, and the associated inflammatory response manifests as cardiorespiratory diseases and respiratory morbidity in children, especially in poor socioeconomic areas where the population is dependent on burning of biomass and solid fuels for heating and cooking, overcrowding and exposure to second hand tobacco smoke. Individuals who are more prone to developing the PM associated manifestation are those who spend lengthy periods of time near the source of solid fuel combustion, such as women and children.[6,8,9]

The human respiratory system has 5 stages of development, with the last stage ranging from 37 weeks of gestation to approximately 20 years of age. Children exposed to air pollution and second hand tobacco smoke are more susceptible to the effects due to behavioral and physiological factors. For example, higher rates of mouth breathing have been observed in children, which is increased by 20% during increased physical activity. Minute ventilation rate in relation to body size coupled with ineffective PM removal by the upper respiratory tract due to increased mouth breathing leads to an increased amount of PM deposition in the lungs. In addition, exposure to environmental tobacco smoke has been linked with an increased prevalence of lower respiratory tract infections such as asthma, bronchitis, bronchiolitis and pneumonia. Adverse changes in lung function related to $PM_{2.5}$ and UFP therefore present at an earlier stage in children. [10–12] Interestingly, it has also been described that the overall lung health of children improved in California when the federal state emission regulation reduced output of motor vehicles, ships and refineries thus reducing $PM_{2.5}$ by 50%.[13]

1.1.4 Impaired alveolar macrophage function in relation to clearance and retention of particulate matter and ultrafine particles.

The alveolar macrophage is derived from bone marrow monocytes and is a component of the mononuclear phagocytic system and forms part of the adaptive immune response. Tissue

macrophages are primarily involved in the processing and transfer of antigens to lymphoid receptors compared to alveolar macrophages. Its principal role is to ensure that alveolar surfaces are void of exogenous agents by phagocytosing foreign material, thus protecting the lung from inhaled substances (including bacteria) which evade mucociliary clearance and potentially cross the epithelial barrier to the pulmonary interstitium.[7,14] Alveolar macrophages phagocytose particles in an opsonin-dependent manner using scavenger type receptors before migrating to the bronchoalveolar junction where accumulation and aggregation occur. High burdens of UFP's however result in increased PM number coming into contact with respiratory epithelium.[8]

Numerous animal models have been used to demonstrate the relationship between the toxicology of PM and the clearance and retention thereof in the lung. It was proposed by *Morrow et al*[15] that the impairment of particle clearance is attributed to the volumetric loading of alveolar macrophage. He suggested that impaired phagocytosis occurs when the composite phagocytosed volume exceeds 6% and that complete cessation of clearance happens at 60%. Both clearance mechanisms and migration of alveolar macrophages have been shown to be impaired as a result of PM exposure.[8,15,16] An impairment of clearance was demonstrated at 2.6% of phagocytosed volume with emphasis placed on UFP.[10,17] Under increased and continued PM and UFP loading, clearance thereof is slowed, this is observed in sputum samples of ex-mine workers with a halftime of approximately 5 years, and the chronic build up may lead to inhibition of the phagocytic function of the macrophage.[14,18–20]

1.2 Benign paediatric pulmonary disease.

1.2.1 Chronic cough vs. chronic wheeze.

A cough is a normal protective physiologic reflex aiding in the clearance of both airway secretions and aspirated material. A cough may be the only presenting symptom of a more serious underlying condition and becomes abnormal when the frequency and severity affects the child's ability to eat, sleep, play or learn.

Most coughing episodes in children are acute, lasting approximately <3 weeks, and are secondary to a lower respiratory tract infection. A cough with a duration of 3-8 weeks is classified as a prolonged acute cough, whilst a cough persisting longer than 8 weeks is

classified as chronic cough. In addition to duration and the likeliness of an underlying condition, the quality and pattern may also be assessed with regards to wet; moist; productive and dry.[21,22]

A physician faced with a child who has a non-specific symptom, such as a chronic cough is faced with placing the child into one of the following categories:

- Normal child.
- Overestimation of symptoms including psychogenic.
- A child with asthma syndrome.
- A child with a serious illness (Tuberculosis or cystic fibrosis).
- A child with a non-serious treatable cause of cough with our without a wheeze (postnasal drip or gastro-oesophageal reflux disease).[23]

A wheeze is defined as continuous high-pitched sounds possessing a musical quality from the chest on expiration. A wheeze should be confirmed by a physician as they can also determine the severity of the wheeze.[24] Fifty percent of wheezing is observed in children of preschool age, <6 years of age. If a wheeze is recurrent, it and may be classified as typical or atypical. Conditions such as asthma; bronchitis and bronchiolitis are associated with typical wheeze. Conditions such as recurrent aspiration, cystic fibrosis and tuberculosis are associated with atypical wheezing.[25]

1.2.2 Serious underlying conditions associated with a typical or atypical chronic cough and wheeze.

Neonatal onset of cough may be suggestive of primary cilia abnormality; lesions compressing the airways; bronchiolitis or chronic viral pneumonia.

1.2.2.1 Asthma: Associated with exposure or situation and may be categorized as acute or chronic. Triggers which cause a hypersensitivity reaction and airway obstruction include exercise; bacterial or viral infection; stress; medications and inhaled allergens and irritants such as dust; particulate matter; animal dander and fumes.

1.2.2.2 Chronic bronchitis: Associated with a chronic cough and sputum production for more than 6 months.[26]

1.2.2.3 Gastro-oesophageal reflux disease: Associated with organizing pneumonia and Reflux aspiration may be associated with or without gastric content.[27]

1.2.2.4 Bronchiectasis: Defined morphologically as the irreversible, abnormal dilation of bronchi and bronchioles. Mainly related to an underlying condition of mucostasis and chronic infection (particularly Tuberculosis in the South African context).[28]

1.2.2.5 Pulmonary tuberculosis: Caused by the acid-fast lipid rich *Mycobacterium tuberculosis* and is spread via airborne droplet nuclei 1-10µm in size. Once phagocytosed, tubercle bacilli replicate within the cytoplasm of alveolar macrophages and spread to the lymphatics and bloodstream and spread to distant organs. When the primary focus in the lung undergoes calcification and fibrosis, it is referred to as the Ghon's lesion.[29]

1.2.2.6 Bronchogenic cysts: Congenital cysts found predominantly in the anterior hilum or mediastinum. Cyst fluid is found within a thickened fibrous wall and may be clear or infected, containing pus and haemorrhage.[30]

1.2.2.7 Primary ciliary dyskinesia: An autosomal-recessive condition causing the primary abnormality of the axoneme dynein arms which drive the beating of cilia. Sixty five percent of patients have a shortening or absence of the outer dynein arm resulting in an ineffective mucociliary escalator thus increased infection and bronchiectasis.[30]

1.2.2.8 Cystic fibrosis: An autosomal-dominant condition resulting in an amino acid deletion of phenylalanine of the cystic fibrosis transmembrane conducting regulator gene on chromosome 7. The protein encoded by the mutant gene resides on the apical membrane of epithelial cells causing decreased permeability of chloride transport across epithelial membranes. The correct composition of water and electrolytes in epithelial secretions is altered resulting in thick mucus accumulation. This is accompanied by repeated infections with acute inflammation in the lungs. Patients with cystic fibrosis are prone to recurrent chest infections and bronchiolitis due to distended mucus glands with inspissated mucus in the airways. A relatively simple sweat test is a good screen test for this condition.[30]

1.2.2.9 Pneumonia: Presents as an acute illness. May be acquired through inhalation or aspiration. It may be either infective (bacterial, viral, fungal or parasitic) or non-infective (chemical) in eitiology.[31]

1.2.3 Underlying conditions in relation to particulate matter and ultrafine particle exposure.

Asthma is characterized by airway obstruction with an increase in alveolar volume resulting from air trapping. It would be expected that there would be enhanced PM diffusional deposition associated with increased alveolar volume. Those therefore suffering with asthma appear to have exacerbations when exposed to air pollution. Increased levels of air pollution are not only associated with asthma exacerbations, but also increased hospital admissions.[7,32]

One such study estimated the risks of various types of $PM_{2.5}$ on hospital admissions for respiratory diseases among children.[33] Elemental and organic carbon, various metals and silicone were measured as components are found in biomass burning; diesel and motor vehicle emissions and other fuel combustion processes. Their study revealed that hospital admissions for childhood respiratory diseases, especially pneumonia, were associated with $PM_{2.5}$ exposure. Another study explored the association between environmental carbon and lower lung function in schoolchildren.[12] Fifty five asthmatic and forty non-asthmatic children were included in this study. Their findings revealed that the association between environmental carbon and decreased lung function was seen in both asthmatic and non-asthmatic children.

The effect of environmental tobacco smoke has also been described to be associated with increased incidences of wheeze up to the age of 6 years of age with an odds ratio of 1:31. Children in smoking households have an increased incidence of cough, phlegm production, bronchitis and bronchiolitis, and thus are associated with increased hospital admissions.[11,25]

1.3 Clinical utility of BALF differential cell counts, lipid-laden and haemosiderin-laden macrophage index.

1.3.1 The diagnostic value of bronchoalveolar lavage fluid cytology differential counts.

In normal individuals 80-90% of cells recovered from BALF are macrophages, followed by 5-15% lymphocytes, less than 3% neutrophils and less than 1% eosinophils.[34] Limited data exists about the cellular components found in bronchoalveolar lavage fluid of normal children due to ethical reasons as this procedure is not normally performed on normal children, only on children who have pulmonary pathology warranting further bronchoscopic investigation. One study using 50 children aged 3-16 years, undergoing elective surgery for non-pulmonary illnesses found that macrophages were the predominant cell type followed by lymphocytes. Thisstudy also revealed that there was a higher granulocyte count in younger children with the neutrophil count being higher than that of eosinophils and basophils.[35] In conditions such as idiopathic pulmonary fibrosis, there is no specific diagnostic feature; however the differential count may provide useful information. In this case, neutrophils are the main increased cell type, and the combination of neutrophils and eosinophils occur in two-thirds of IPF patients.[36]

Below are the various differential cell count patterns and associated percentages which are indicative of various lung diseases.[34,37,38]

- Lymphocytic patterns with a lymphocyte count greater than 15% are seen in conditions such as non-specific interstitial pneumonia, hypersensitivity pneumonitis and drug-induced pneumonitis. A lymphocytic percentage greater than 50% is highly suggestive of non-specific interstitial pneumonia or hypersensitivity pneumonitis.

- Neutrophilic cell patterns with a neutrophils count of more than 5% are seen in idiopathic pulmonary fibrosis, aspiration pneumonia, bacterial and fungal infection, diffuse alveolar damage and bronchitis. A neutrophil percentage greater than 50% strongly suggests pneumonia, lipid pneumonia, lung abscess or acute lung injury.

- Eosinophilic patterns yielding an eosinophilic count greater than 3% are seen in eosinophilic pneumonia, drug induced pneumonitis, asthma, bronchitis, bacterial infections, parasitic infections and fungal infections. An eosinophilic count greater than 25% is suggestive of an eosinophilic lung disease.[34,37,38]

1.3.2 The role of the lipid-laden macrophage index in exogenous and endogenous lipid.

The accumulation of lipid within alveolar macrophages occurs as a result of phagocytosis of external lipid and lipoproteins. This may also be secondary to endogenous sources as is seen in lipid pneumonia as a result of a chronic lung infection or bronchial obstruction.

Exogenous sources however are as a result of either aspiration or inhalation of lipid.[39] The laryngeal reflex protects the airways from coming into contact with gastric and oral content. Chronic respiratory disease in children may arise as a result of recurrent pulmonary aspiration. Forty to fifty percent of infants with gastro-oesophageal reflux disease (GORD) present with respiratory symptoms such as chronic cough, wheeze and recurrent pneumonia. Numerous tests are used in the diagnosis of GORD, however no single test is considered to be a gold standard test in the diagnosis of chronic pulmonary aspiration. The tests used to diagnose GORD are:

- Oesophageal pH test: An electrode probe is placed in the distal oesophagus where continuous recording the pH is performed. Scores less than pH 6.4 or 3.7%.in the proximal oesophagus and pH 16.1 or 6.2% in the distal oesophagus indicates abnormal oesophageal pH and acid exposure suggesting reflux. [71]

- Barium eosophogram: Barium in the form of an oral fluid is administered. Upper gastro-intestinal (UGI) aspiration is recorded if barium appears in the airway below the vocal cord. UGI direct is observed if aspiration occurs during swallowing and UGI reflux is observed if barium is found to move from the stomach to the oesophagus.

- The lipid-laden macrophage index (LLMI): The presence of alveolar lipid laden macrophages in bronchoalveolar lavage fluid is considered to be a marker for lipid aspiration. Lipid stains such as the oil red O or Sudan black are used to demonstrate phagocytosed lipid in alveolar macrophages. The

amount of lipid as reported by *Corwin and Irwin et al*[40] are graded 0-4.(0= not opacified; 1= $^{1}/_{4}$ opacified; 2=$^{1}/_{4}$ -$^{1}/_{2}$ opacified; 3=$^{1}/_{2}$ -$^{3}/_{4}$ opacified; 4= totally opacified.) a total of 100 macrophages are evaluated and a score ranging of 0-400 is reported.

Our laboratory routinely reports on the LLMI, however, it has been found in many studies that the number of lipid-laden macrophages has a low sensitivity for aspiration, and is often increased in pulmonary disorders unrelated to aspiration. The lipid-laden macrophage index thus cannot independently identify or exclude aspiration. A positive lipid-laden index over estimates the probability that a patient has GORD.[41,42] Other studies investigating the lipid-laden macrophage index in children, known with a history of GORD, had a median score of 41.5% positivity compared to those who did not. Non-GORD children had scores of 12.5% positivity. Patients who scored an LLMI greater than 60% were likely to have aspiration GORD.[39,43]

Advances utilizing cow-milk protein specific immunocytochemical stains demonstrating aspiration in formula-fed infants have been explored. β-Lactoglobulin and α-lactalbumin cytoplasmic positivity in alveolar macrophages have been compared to the oil red O lipid laden macrophage index. α-Lactalbumin was more effective at demonstrating cow milk proteins associated with aspiration in formula-fed infants.[44]

Pepsin, a gastric enzyme, is stored as pepsinogen in gastric chief cells. It is a protease digestive enzyme and when released by the chief cells in response to stimulation, is responsible for breaking down proteins into peptides. Literature suggests that the detection of pepsin in tracheal aspirates and BALF is more reliable in diagnosing GORD and microaspiration in children. Phillipe et al [40] used an immunoassay with rooster polyclonal antibodies to purified human pepsin to study pepsin as a marker in GORD-related pulmonary aspiration. Their finding revealed that pepsin-positive tracheal aspirates were more likely to have a clinical diagnosis of GORD, these children had either a history of chronic respiratory symptoms or both reflux coupled with chronic respiratory symptoms.[40]

Another BALF pepsin study used an enzyme linked immunosorbent assay (ELISA) using monoclonal antibody to porcupine pepsin to detect pepsin in BALF of post lung transplant patients suffering from GORD. Their study revealed that the detection of pepsin in BALF of patients in this group is a useful test to identify those patients at risk for developing GORD-related pulmonary aspiration.[45]

1.3.3 The hemosiderin-laden macrophage index in diffuse alveolar haemorrhage and haemosiderosis.

Pulmonary alveolar macrophages process haemoglobin from phagocytosed erythrocytes to form ferritin. Haemosiderin is demonstrated in alveolar macrophages when stained with a ferrocyanide such as potassium ferrocyanide to form a blue compound. This is a simple diagnostic test performed on bronchoalveolar lavage fluid taken from infants and young children.

The presence of haemosiderin-laden macrophages indicates the presence of haemosiderosis, idiopathic haemosiderosis or diffuse alveolar haemorrhage. These are serious and potentially fatal conditions in children and infants and the diagnosis is suggested by the presence of haematamesis; pulmonary infiltrates and the presence of haemosiderin laden macrophages.[46]

Diffuse alveolar haemorrhage refers to a clinical syndrome characterized by extensive bleeding into the distal airways and alveoli as a result of microvascular injury. This may be associated with other underlying disorders such as infections and drugs such as penicillamine. Haemosiderin-laden macrophages only appear after 48 hours after the start of the bleed. The most commonly employed criterion aiding the diagnosis of diffuse alveolar haemorrhage is a haemosiderin-laden macrophage index score greater than or equal to 20% positivity.[47,48]

Pulmonary haemosiderosis may be seen in association with cow-milk hypersensitivity, Goodpasture's syndrome, aspiration of a sharp object or idiopathic. *Salih et al* [49] investigated the sensitivity and specificity of haemosiderin-laden macrophages in 6 children with a mean age of 4.36 year and a diagnosis of bronchitis, bronchiolitis or haemosiderosis. They were initially diagnosed with haemoptysis and 4 were diagnosed with pneumonia. Those that had pneumonia had a mean haemosiderin-laden macrophage index of 36% positivity for phagocytosed haemosiderin.[46,49,50]

1.3.4 The periodic acid-Schiff test in alveolar proteinosis and interstitial lung disease.

Differential counts performed on bronchoalveolar lavage samples can be ineffective in conditions such as idiopathic pulmonary fibrosis, sarcoidosis and pulmonary alveolar proteinosis.

Alveolar proteinosis is a condition characterized by the progressive filling of the alveoli by surfactant glycoproteins and related products due to imbalanced production and removal of

phospholipids by the type 2 pneumocytes and defective alveolar macrophage function from lamellar body ingestion. This condition may be idiopathic; related to immunosuppression, infection or as a result of chronic exposure to organic dust such as silica. Both extracellular and phagocytosed glycogen and phospholipoproteins contain high concentrations of carbohydrate macromolecules, which stain positively with the periodic acid-Schiff stain. This is seen as bright magenta pink staining proteinaceous material. A positive PAS may be used to assist in the diagnosis of alveolar proteinosis and confirm recurrence or persistence of this condition.[51,52]

Although the stain is used predominantly in the diagnosis of alveolar proteinosis, one study demonstrates its usefulness in interstitial lung diseases. It revealed that known patients with sarcoidosis, idiopathic pulmonary fibrosis and extrinsic allergic alveolitis, demonstrated increased macrophage PAS positivity.[53]

1.4 Cytological preparation, processing and interpretation of paediatric bronchoalveolar lavage fluid.

1.4.1. Bronchoalveolar lavage fluid transport and processing.

One of the recommendations described in literature is that BALF should be transported in containers that do not promote cellular adherence to the container's surface such as glass or certain plastics. Most authors agree that BALF should be transported at room temperature (21ºC) if the transport to the laboratory and processing is less than 60 minutes as cells are not preserved well in saline. If transport is delayed resulting in processing after 60 minutes, the BALF can be stored at 4ºC for up to 24hours.[34,37,54]

It has been suggested that the filtration of BALF through a loose nylon gauze may be a better technique when compared to cytocentrifugation as it yields fewer unsatisfactory specimens and traps large aggregates of mucin, although sputolysins may also be used.[2] Various methods exist for processing and cellular quantification of BALF with regards to the differential count, lipid-laden macrophage index and haemosiderin-laden macrophage index. The cellular profiles of BALF may assist in determining the nature of the pulmonary disease. Manual BALF counting methods use haemocytometers for the initial cell count followed by

differential cell counts being performed on cytospin or cell smear preparations. This however requires trained personnel, is a time-consuming test and is subject to individual interpretation.[55]

One such method for the initial preparation utilizes the filtration of pooled BALF through gauze; however, this may greatly diminish the volume of the sample, resulting in lower cell counts especially in macrophage. *Ratjen et al* [35]used cell culture medium, Eagles minimum essential medium, to maintain the cells' integrity after gauze filtration. BALF cell viability can be assessed using trypan blue staining when counting BALF leukocytes using a haemocytometer. Once quantified on a haemocytometer, BALF undergoes centrifugation at 50-500rpm for 10-15 minutes for cell subtype differential counts on Diff-Quick® stained slides using manual counts on simple smears. Lymphocyte, macrophages, neutrophils and eosinophils are ultimately expressed as a percentage per 100 cells counted in manual differential counts on BALF. More than one count is often performed and in a quest for maximum accuracy of differential cell count median percentages, some authors used a total of 300-350 cells in their differential count whereas others used a total of 600 cells per subject in their differential count to attain maximum median accuracy per individual differential count cell type.[34,35,56]

1.4.2 Manual cell counts vs. electronic cell counts.

Apart from the extensive time taken to perform manual counts on a haemocytometer, other sources of error described include:

- Variations in the BALF sample haemocytometer loading volume
- Haemocytometer chamber filling speed
- Cell aggregates failing to enter the chamber
- Cell distribution within the haemocytometer.

The coulter counter, originally developed for counting blood cells, is an automated cell counting method which quantifies the number of cells within a conductive solution and determines their size when passed through a small orifice using changes in electrical conductance. The level of deflection is seen on a meter. This is the basis of modern day flow cytometry. Studies have been performed comparing manual haemocytometer counts to that of

electronic cell counting to measure total cell numbers in BALF using an automated Coulter® Counter D industrial model. The study revealed better reproducibility and faster results with an electronic counter compared to manual counts by experienced observers.[57,58]

1.4.3 Quantitation of the lipid-laden macrophage index and the haemosiderin-laden macrophage index.

Numerous studies employed the Golde score for the quantification haemosiderin-laden macrophages first described by Colombo and Hallberg for lipid-laden macrophages[42,47,59–63] where 200-300 alveolar macrophages were counted and graded 0-4 according to the amount of phagocytosed lipid/haemosiderin dividing the number of lipid/haemosiderin-laden macrophages by the total number of cells counted.

- Grade 0 had no blue/red colour n the cytoplasm. (no opacification)
- Grade 1 had faint blue/red cytoplasmic staining ($^1/_4$ opacification)
- Grade 2 had either dense blue/red staining in a portion of the cytoplasm or medium colour intensity throughout the cytoplasm. ($^1/_4$ -$^1/_2$ opacification)
- Grade 3 had a deep blue/red staining throughout the cytoplasm. ($^1/_2$-$^3/_4$ opacification)
- Grade 4 was entirely intensely blue throughout the cytoplasm. (totally opacified)

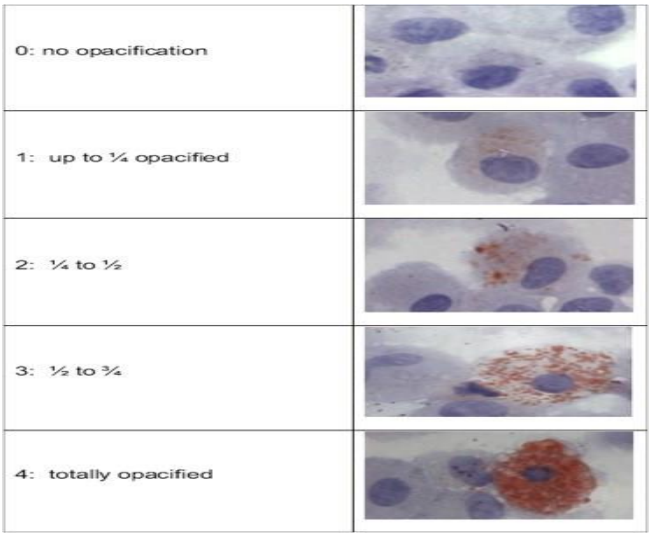


Figure 1.1: Colombo and Hallberg score for lipid laden macrophages. (https://ars.elscdn.com/content/image/1-s2.0-S0954611113004150-gr1.jpg)

Hemosiderin content of macrophages	Number of cells	Coefficient x $n°$ of cells
Type 0	n^0	$0 * n^0$
Type 1	n^1	$1 * n^1$
Type 2	n^2	$2 * n^2$
Type 3	n^3	$3 * n^3$
Type 4	n^4	$4 * n^4$
Total Score (for all evaluable cells, n^{tot})		Sum^{tot}
Golde Score (extrapolated to 100 cells)		$Sum^{tot} / n^{tot} * 100$

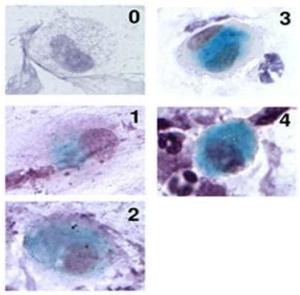


Figure 1.2: Golde score for haemosiderin-laden macrophages. https://www.researchgate.net/profile/Hans_Stricker/publication/269717617/figure/fig4/AS:341460927959044@1458422037177/Description-of-the-Golde-score-calculation-in-sputummacrophages.pnn

Chapter 2: Aim and study design

2.1 Aim.

a) To determine if the BAL specimens can be used to categorize children with wheezing into either a gastro-eosophageal reflux or primary respiratory pathology group. Differential cells counts and counts of Oil red O positive lipid-laden macrophages, as a marker of the gastro-eosophageal reflux and pulmonary aspiration. Perl's Prussian blue positive haemosiderin-laden macrophages for pulmonary haemosiderosis and carbon-laden macrophages were used as marker of pulmonary pathology.

b) To determine if BAL can assist with the sub-classification of the primary respiratory pathology group.

c) To determine if the amount of phagocytosed carbon by alveolar macrophages corresponds to the patient's environment of increased pollution and if these children have an increased risk for lung disease.

2.2 Hypothesis.

Alveolar fluid collected via the BAL technique is often abundantly cellular.

a) The BAL cell counts will reliably distinguish the children that suffer from GERD from those who do not.

2.3 Study design and case selection.

This was a prospective study that enrolled consecutive, consenting patients who underwent a flexible bronchoscopy at Tygerberg Academic Hospital with BAL procedure for the workup of wheezing.

Patients with cystic fibrosis and positive pulmonary tuberculosis infections where excluded.

All samples were received in 12ml plastic test-tubes. A minimum of 2mls and maximum of 10mls of fluid were collected. All samples were processed as part of the routine service

provided by NHLS cytology; Tygerberg Hospital by cytotechnologists trained to process and perform paediatric BALF differential special stain cell counts. Samples were checked for adequacy by performing a routine haemocytometer count on unfiltered BALF. Samples with a total cell count of less than 50 cells/ml were rejected and not included in the statistical analysis in the study as there would have been too few cells present to perform differential cell counts and special stains. Adequate Samples were reviewed by cytotechnologist, Miss M. Marshall and pathologist, Professor P. Schubert.

2.4 Diagnostic categories.

After a full clinical work-up, the patients who originally presented either with a chronic wheeze, recurrent lower respiratory infection or both were given a final diagnosis. This was placed into 2 broad categories by the study's collaborating senior paediatric pulmonologist at Tygerberg hospital, Professor P. Goussard. which were:

- Recurrent wheeze.
- Recurrent infection.

These 2 broad categories of chronic wheeze and recurrent infection were further sub-divided into 6 sub-categories based on their final diagnosis after a full clinical workup.

These sub-categories were:

- Alveolar proteinosis
- Gastro-oesophageal reflux disease
- Idiopathic pulmonary haemosiderosis:
- Infection/ inflammation These patients had a final outcome of either pulmonary tuberculosis; recurrent lower respiratory tract infection; pneumonia or bronchiolitis obliterans
- Structural abnormality: These patients had a final outcome of either persistent right lower or middle lobe collapse; subglottic stenosis; laryngomalacia;; bronchogenic cyst; innominate artery compression or vascular airway compression.
- Idiopathic wheeze: These patients had no specific diagnosis after a full clinical investigation for their condition thus resulting in a final diagnosis of idiopathic recurrent wheeze.

The patients in the study were categorised into main and sub-categories for the study to ascertain if the laboratory tests could serve as a marker of pulmonary pathology; determine if

patients who presented with a recurrent wheeze could be placed into a pathological group and if the amount of environmental carbon significantly contributed to their condition in our setting based on literature.

This flow diagram breakdown can be seen in the figure 2.1..

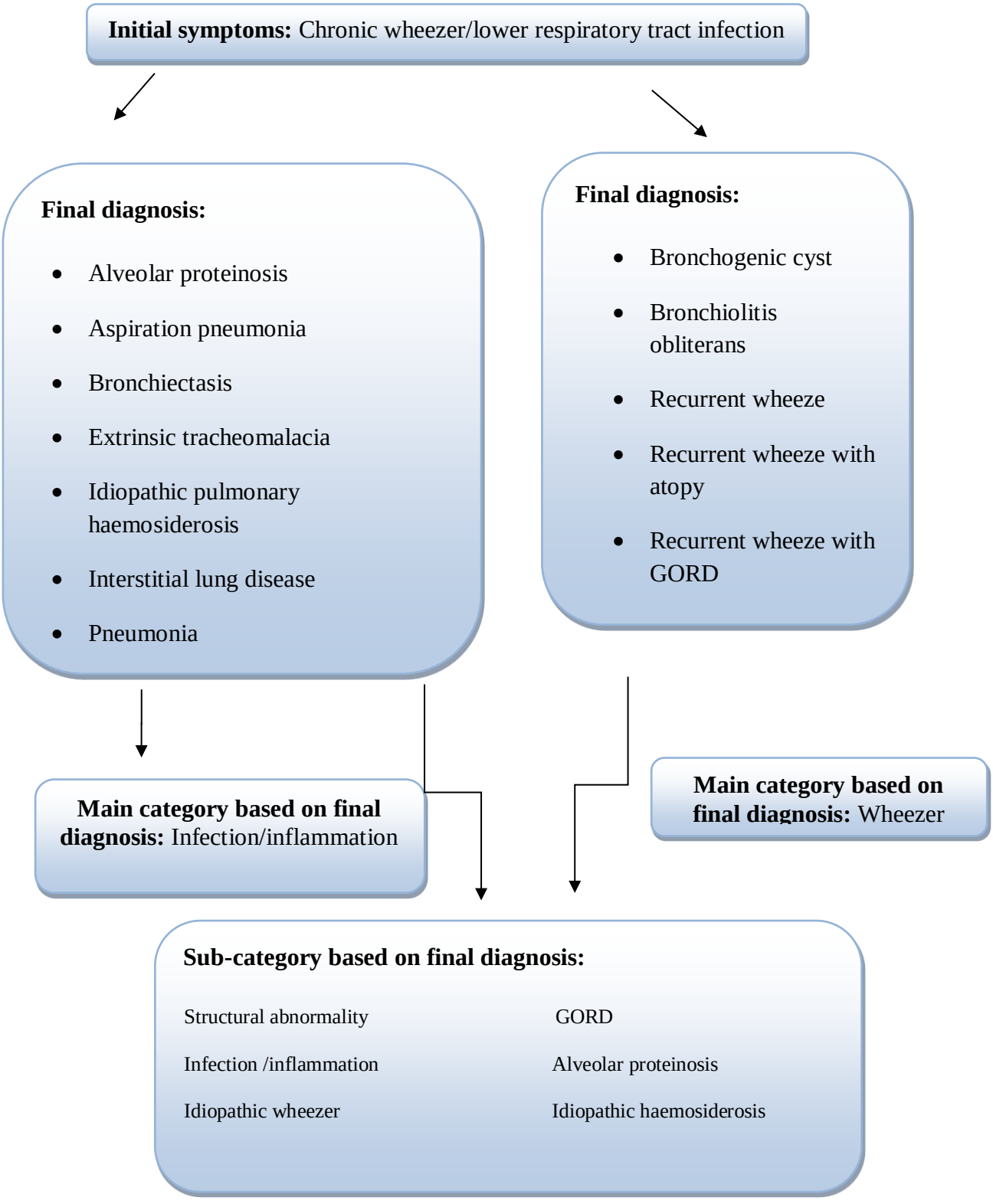


Figure 2.1 Final diagnostic outcomes, diagnostic main categories and sub-categories.

2.5 Laboratory investigations.

The final clinical diagnosis was made after the entire workup was completed. The following tests were performed on the BALF and serum in this group of children to formulate a final outcome.

Histological staining technique

- Perl's Prussian blue
- Periodic acid-Schiff

Cytology investigations: BALF specimen

- Differential cell count
- Carbon-laden macrophage quantification
- Lipid laden macrophage index • Haemosiderin laden macrophage index
- PAS positivity.

MTB investigations: BALF specimen

- GeneXpert MTB/RIF Ultra assay
- Mycobacterium Tuberculosis culture.
- Auramine O fluorescent stain for acid-fast bacilli.

Microbiological investigations: BALF specimen

- Gram stain.
- Microbiological culture.

Chemical investigations: Sweat and serum.

- SWEAT test.
- Serum IgA.
- Serum IgG.
- Serum IgM.

Virology investigation of BALF:

- Respiratory virus multiplex.
- Cytomegalovirus

Statistical analysis of the laboratory findings of the broad wheezer category will be compared against the broad categories of infection/inflammation, as well as the sub-categories of structural abnormality; GORD; infection/inflammation; idiopathic wheezer; alveolar proteinosis and idiopathic haemosiderosis.

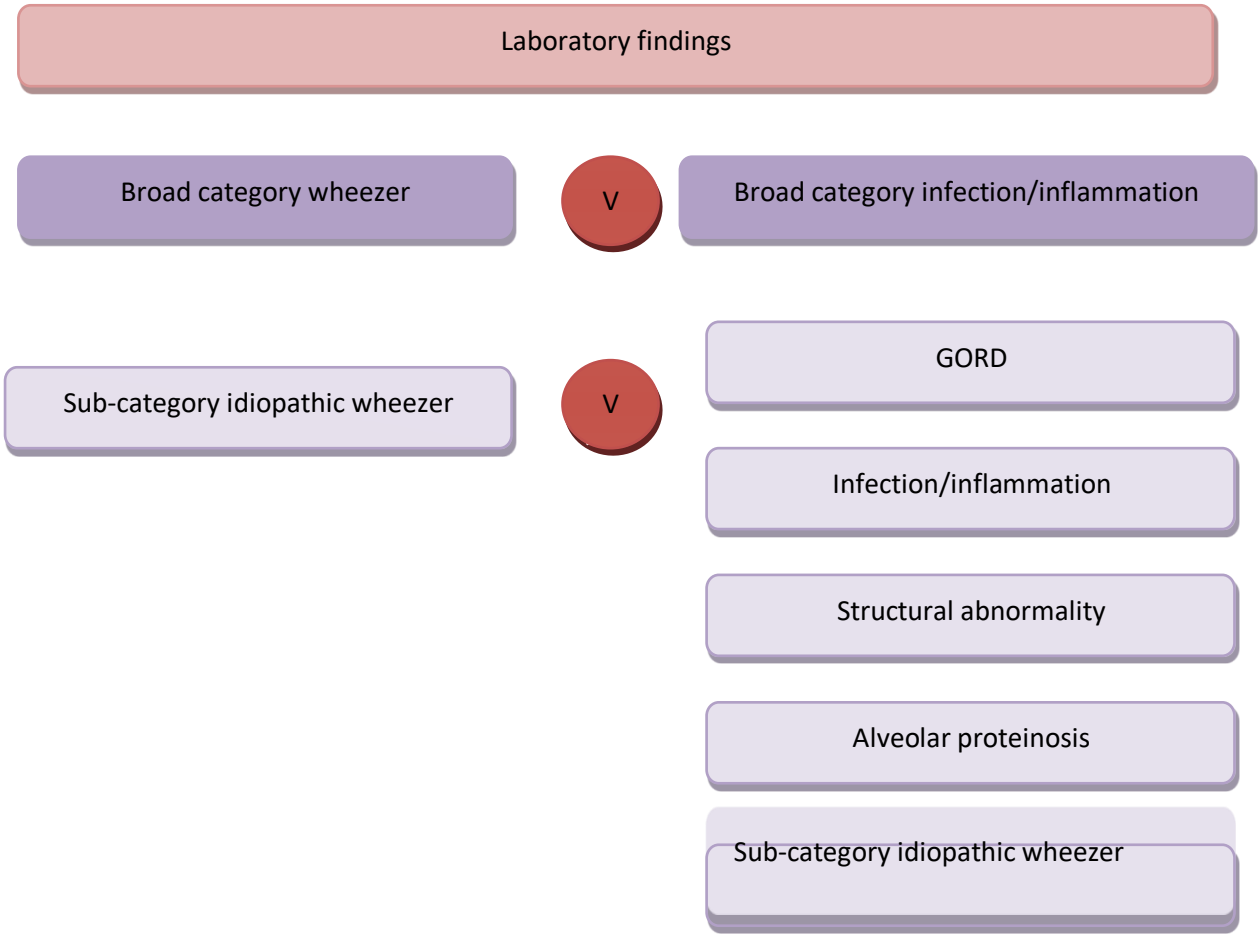


Figure 2.2: Final diagnostic main category and sub-category comparisons of laboratory findings.

2.6 Cytology differential count and special stain grading system.

All processing of paediatric bronchoalveolar lavages was performed using a method developed by Dr CC Chase and her supervisor Professor D.J Roussouw at the University of Stellenbosch department of Anatomy and Histology. This method has been used at NHLS Cytology since 2007 at Tygerberg hospital. (Appendix 1,22-24)

Samples were checked for adequacy by performing a routine haemocytometer count on unfiltered BALF. Samples with a total cell count of less than 50 cells/ml were rejected as there would have been too few cells present to perform differential cell counts and special stains.

Adequate samples were processed for differential cell counts, lipid-laden macrophage index(LLMI), haemosiderin-laden macrophage index (HLMI) and carbon-laden macrophages (CLM) and quantified accordingly.

2.7 Ethical approval.

Ethical approval was obtained from the Health Research Ethics Committee, Stellenbosch University. Project ID 2517 (S16/08/148).

Chapter 3: Materials and methods

3.1 Paediatric bronchoalveolar lavage fluid collection.

Flexible bronchoscopy and collection of BALF was performed by the division of paediatric pulmonology, Department of Paediatrics and Child Health, Tygerberg Hospital, Stellenbosch University, under the guidance of Professor P. Goussard.

The bronchoalveolar lavage fluid was obtained by using 10-20ml aliquots of sterile saline warmed to body temperature (37-38ºC) and lavaging the distal airspaces before re-aspirating the fluid as per the ERS task force guidelines for bronchoalveolar lavage in children.[64] The instilled fractions will be retrieved and pooled in a 12ml plastic test-tube and sent to the cytology department for routine cytological analysis.

3.2 Cytology processing of bronchoalveolar lavage fluid.

We have been processing paediatric bronchoalveolar lavages since 2007 as per the method pioneered by Professor D.J Roussouw and Dr. CC Chase and her supervisor at Stellenbosch University, Department of Anatomy and Histology. Unstained leukocytes in unfiltered BALF are manually counted in a haemocytometer to check for adequacy and necessary dilution if required. Cytospin slides are then prepared and slides are stained using the Papanicolaou; Giemsa; Oil red O in cytology. Cytospin slides destined to be stained for haemosiderin and glycoproteins are performed by histology. Differential cell counts, lipid laden macrophages and haemosiderin-laden macrophages are then quantitated and reported accordingly. Their work was never published; however, her method was presented the 37th Annual Conference of the Anatomical Society of Southern Africa, 2007.22-25 April. (Appendix 1, Appendix 22-24)

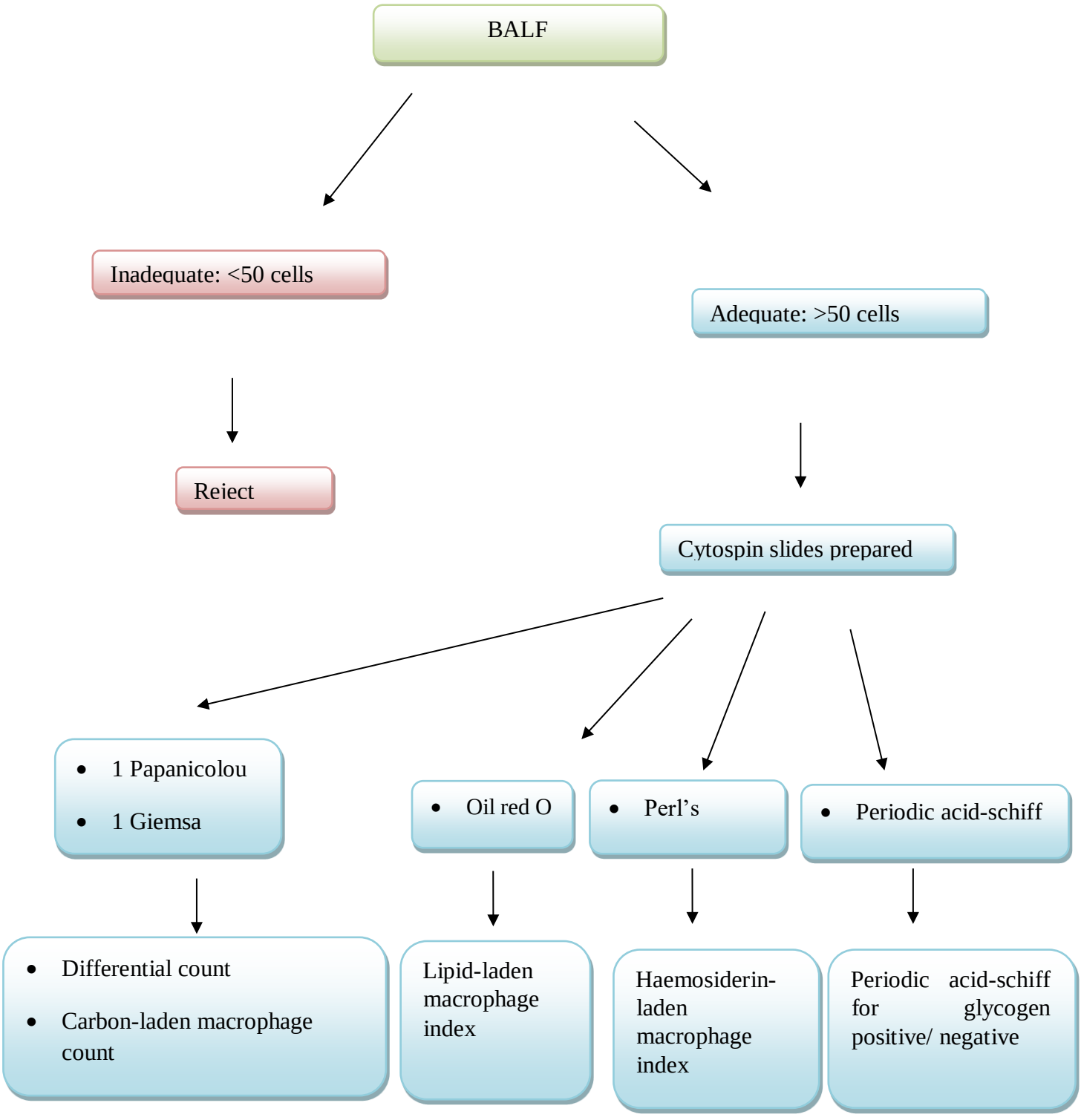


Figure 3.1: Illustration of paediatric BALF specimen cytology processing.

3.2.1 Specimen adequacy and haemocytometer manual cell counts.

The entire counting chamber of a haemocytometer is 3 x 3mm with a depth of 0.1mm (Appendix 22). The area of the counting chamber is therefore 9mm^2 with a depth of 0.1mm. When converting the number of cells to volume per ml after counting a full chamber, the average of the 2 counts are multiplied by 0.9 (area x depth) and multiplied by 1000 (10^3) to express the cell quantity per ml. The same applies to a half chamber count in a slightly more cellular smear, with the exception of using half of the aforementioned area but still keeping the chamber depth unchanged. If there is a very cellular specimen, only the central 25 squares are counted. The central 25 square has an area of 1 x1mm, the total number of cells therefore counted in the central 25 squares must be divided by 0.1 to get the total number of cells per ml.

If using a hypothetical average cell count of 50 as an example used in each chamber count, the calculations would be explained as follows:

- Full chamber:

$$\text{Cells/ml} = \frac{\text{Average amount of cells counted}}{\text{Chamber depth x chamber area}}$$

$$= \frac{50}{0.1 \text{ x } 3 \text{ x } 3\text{mm}}$$

$$= \frac{50}{0.9\text{mm}^3}$$

$$= 55.56\text{x}10^3 \text{ cells/ml}$$

- Half chamber:

$$\text{Cells/ml} = \frac{\text{Average amount of cells counted}}{\text{Chamber depth x chamber area}}$$

$$= \frac{50}{0.1 \text{ x } (0.5 \text{ x } 3 \text{ x } 3\text{mm})}$$

$$= \frac{50}{0.45mm^3}$$

$$= 111.11x10^3 \text{ cells/ml}$$

- Central 25 squares

$$\text{Cells/ml} = \frac{\text{Average amount of cells counted}}{\text{Chamber depth x chamber area}}$$

$$= \frac{50}{0.1 \text{ x } 1 \text{ x } 1mm}$$

$$= \frac{50}{0.1mm^3}$$

$$= 500x10^3 \text{ cells/ml}$$

More cells require less volume of BALF when making cytospin smear. In addition, depending on the total cellularity, appropriate dilutions are required to be made in order to achieve an even distribution of cells on the cytospin.

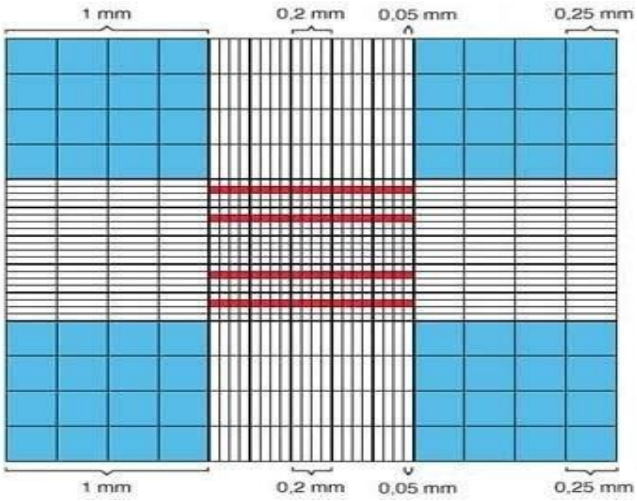


Figure 3.2: Haemocytometer counting chambers. (https://www.researchgate.net/profile/Aamir_Khan6/publication/230815213/figure/fig2/AS:340610247610371@1458219219805/2-Schematic-representation-of-the-Haemocytometer.png)

Method:

1) BAL laboratory number and volume was measured and recorded in a BAL book

2) A small amount of unfiltered BALF was decanted into a separate tube if specimen was more than 8ml.

3) Two separate cell counts were performed on unfiltered BALF if less than 8ml on a haemocytometer as follows.

- Macrophages (granulocytes were counted with macrophages), lymphocytes, red blood cells and columnar epithelial cells were counted.

- Full chamber: If there are 1-2 cells per square (or fewer) in the haemocytometer, excluding red blood cells and epithelial cells. All 169 squares were counted, divided by 0.9 and multiplied by 10^3.

- Half chamber: If there were more cells, fewer squares were counted. The total macrophage and lymphocyte count was at 100, fewer cells were counted. The count would start at the middle line and the result was divided by 0.45 and multiplied by10^3.

- Central 25: If the sample was too cellular, only the central 25 squares were counted. The result was multiplied by 10 x10^3

4) The specimen was fixed with an equal volume of BAL suspension and histological phosphate buffered formalin as follows:

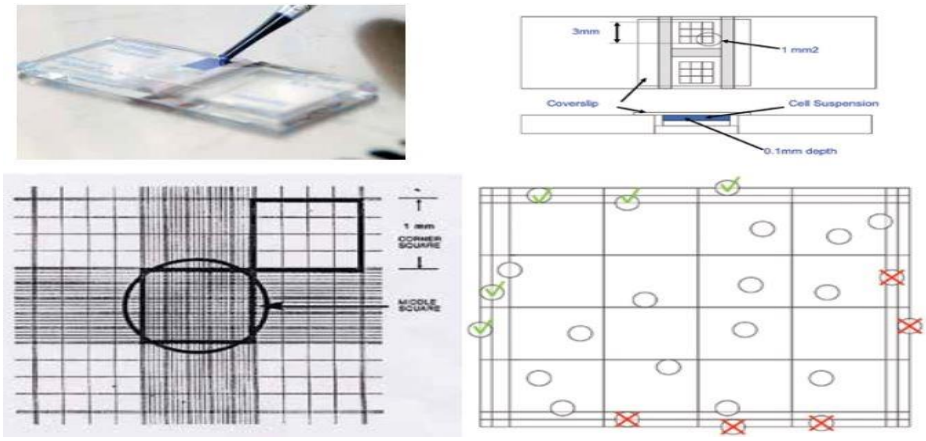


Figure 3.3: Loading of specimen and quantitation of cells using a haemocytometer.

(*https://www.sigmaaldrich.com/content/dam/sigmaaldrich/articles/protocols/biology/subculture-of-adherent/Cellcounting_2.JPG*)

Table 3.1: Manual haemocytometer count requiring no dilution.

Unfixed: Cells/ml (x10³)			Fixed: cells/ml(x x10³)		
	Drops		µl	Papers	smears
Before Fixation	*After fixation*				
	1-24	14	-		4
50-60	25-30	14	434	2	2
60-80	30-40	13	403	2	2
80-100	40-50	11	341	2	2
100-120	50-60	9	279	2	2
120-160	60-80	7	217	2	2
160-240	80-120	5	155	1	2
240-300	120-150	4	124	1	2
300-380	150-190	3	95	1	2
380-440	190-220	2	90	1	2

Samples with these cell counts are fixed with equal volumes of BALF and buffered formalin.

s Samples with 1-24x103(very low counts): take 8m filtered BALF, add 2ml formalin.

Source: Dr CC Chase, department of Anatomy and Histology, University of Stellenbosch. 2 Oct 2007.

Table 3.2: Manual haemocytometer count requiring a 4x dilution.

440000-880000 cells/ml: dilute with normal saline.

Unfixed: Cells/ml (x10³)			Fixed: cells/ml(x x10³)		
	Drops		µl	Papers	smears
Before Fixation	*After fixation*				
440-460	110-115	5	155	1	2
460-600	115-150	4	124	1	2
600-740	150-185	3	95	1	2
740-880	185-220	2	90	1	2

4x Dilution using 2ml saline+4ml formalin+2ml BAL

Source: Dr CC Chase, department of Anatomy and Histology, University of Stellenbosch. 2 Oct 2007. *Table 3.3: Manual haemocytometer count requiring a 8x dilution.*

880000-2000000 cells/ml: dilute with normal saline as follows:

Unfixed: Cells/ml (x10$_3$)			Fixed: cells/ml(x x10$_3$)		
	Drops		µl	Papers	smears
Before Fixation	*After fixation*				
880-920	110-115	5	155	1	2
920-1200	115-150	4	124	1	2
1200-1600	150-200	3	95	1	2
2000-2400	200-300	2	90	1	2

8x Dilution using 3ml saline+4ml formalin+1ml BAL

Source: Dr CC Chase, department of Anatomy and Histology, University of Stellenbosch. 2 Oct 2007.

3000x10^3: 280µl BAL+ 2ml saline+ 2ml formalin (mixed before adding BALF). Use 90 µl

4000x10^3: 200µl BAL+ 2ml saline+ 2ml formalin (mixed before adding BALF). Use 90 µl

5000x10^3: 160µl BAL+ 2ml saline+ 2ml formalin (mixed before adding BALF). Use 90 µl

6000x10^3: 133µl BAL+ 2ml saline+ 2ml formalin (mixed before adding BALF). Use 90 µl

7000x10^3: 144µl BAL+ 2ml saline+ 2ml formalin (mixed before adding BALF). Use 90 µl

8000x10^3: 100µl BAL+ 2ml saline+ 2ml formalin (mixed before adding BALF). Use 90 µl If fixed cells can't be counted, there should be about 200x10^3 per ml in the entire chamber.

5) Unfixed BALF with a total cell count of <50 in the haemocytometer were rejected as too few cells would be present to perform the differential count and special stains.

6) Fixed BAL was allowed to stand for 10 minutes at room temperature.

7) If staining could only be performed later, the fixed suspension was placed in the refrigerator.

8) If, however, the total count could not be performed on the same day, the specimen was fixed with equal volume of buffered formalin and stored in the refrigerator. Dilutions, if required were then performed on the fixed specimen

3.2.2 Cytocentrifuge slide preparation

Method:

1) The laboratory tracking number, name and stain was written on the slide.

2) The cytospin apparatus was assembled in a biosafety cabinet by placing the slide, plastic funnel and relevant number of filter cards in the cytospin holder ensuring that the holes in the filter card and plastic funnel coincide at the back of the cytospin holder and ensuring that the filter paper has it's indentations facing upwards.

3) The fixed BAL specimen was re-suspended using a 3ml plastic pipette.

4) The recorded volume of BAL fluid was pipetted into the funnel.

5) The cytospin bucket was loaded, balanced, sealed and placed into the cytocentrifuge.

6) The centrifuge was set to spin for 7 minutes at 1350rpm.

7) Once centrifugation was completed, the cytospin bucket was removed from the cytocentrifuge and returned to the biosafety cabinet.

8) Cytospin apparatus was disassembled and the slides were allowed to dry before being stained with the Papanicolaou; Rapid-Diff; Oil-red O; Perl's Prussian blue and periodic acid-Schiff stains.

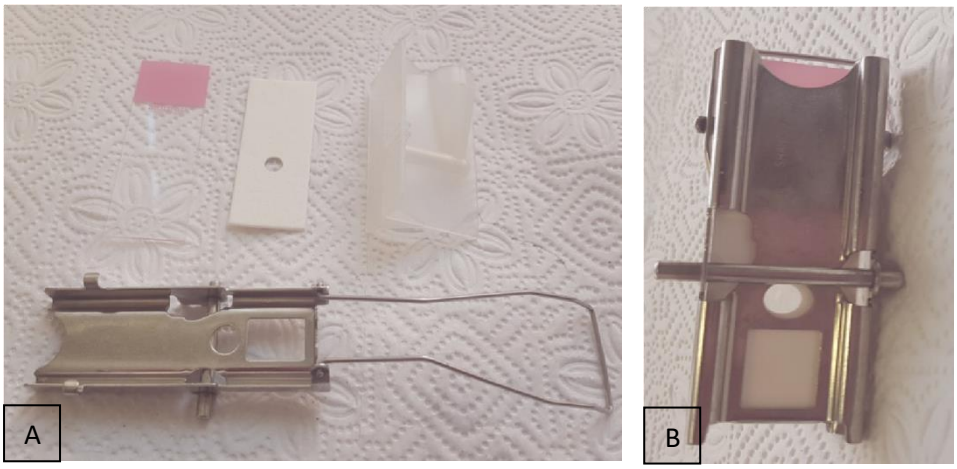


Figure 3.4: (a)Cytospin apparatus unassembled. (b)Cytospin apparatus assembled with coinciding filter and funnel holes.

3.2.3 Papanicolaou staining technique.

The Papanicolaou staining method is a polychromatic staining reaction used to display cellular morphology by demonstrating various degrees of cellular maturity, metabolic activity and the variations thereof on spray-fixed slides. The staining method consists of a nuclear stain and 2 cytoplasmic counterstains. Hydration prepares the cells to take up nuclear stain and dehydration prepares the cell to take up the counterstains. One of 2 methods can be used when staining cells with the Papanicolaou stain. The first is the progressive method where the nucleus of the cell is stained with haematoxylin, the nuclear dye, until the desired staining intensity is achieved and a blueing agent such as lithium carbonate is placed shortly after the haematoxylin step to allow the nuclear stain to set without tinting the cytoplasm. The other method is the regressive method where the nucleus is overstained with a non-acidified haematoxylin and a dilute solution of hydrochloric acid (HCL) is placed after the nuclear stain step thus removing excess haematoxylin. The cells are briefly exposed to this decolourising acid and then rinsed in water to prevent hypochromasia. The aluminium sulphate in the dye ensures that haematin the haematin can bind to the phosphoric groups on the DNA chain by supplying positively charged ions. OG or Orange G is an acidic dye used to stain keratin intensely orange. The stain is also taken up by the eosinophilic granules in superficial cells. EA is a polychrome stain and is the combination of light green SF yellowish and eosin Y. The light green SF yellowish is an acidic dye which is involved in staining the cytoplasm of metabolically active cells such as parabasal cells, leukocytes, histiocytes and

columnar cells. Eosin Y is also acidic and is responsible for staining cilia, erythrocytes, nucleoli and the cytoplasm of superficial squamous cells.[65]

The staining method employed in our laboratory is regressive. Harris haematoxylin, EA-50, OG II,32% hydrochloric acid (HCL), lithium carbonate ($LiCO_3$) , methanol, Rapidiff 1 and Rapidiff 2 are commercially obtained for routine laboratory use. (Appendix 2)

A lithium carbonate saturated stock solution is prepared by dissolving commercially bought pure $LiCO_3$ tablets in distilled water, the supernatant is used to make up the working solution.

Working solutions are prepared for use in the Papanicolaou staining method, namely the hydrochloric acid (HCL) working solution and Li_2CO_3working solution.

Results:

Nuclei: Basophilic

Cytoplasm:

- Superficial cells: Eosinophilic
- Intermediate cells: Cyanophilic/Eosinophilic
- Columnar and non-epithelial cells: Cyanophilic
- Hyperkeratotic and parakeratotic cells: Orangeophilic

3.2.4 Giemsa staining technique.

The Giemsa stain is a staining procedure performed on air-dried slides. The Rapi-Diff stain is the modified rapid version of the giemsa stain. Both require fixation in methanol as the initial step to prevent cells from having flat nuclear patterns which may pose diagnostic difficulties. A useful stain used in the demonstration of non-epithelial components such as myxomatous material, mucin, amyloid and colloid or where haematological disorders may be suspected. (Appendix 3)

Results:

- Nuclei: Purple
- Cytoplasm: Pink/blue
- Eosinophils: Pink/red
- Mucin/amyloid/myxomatous material: Bright pink.

3.2.5 Oil red O staining technique.

The Oil red O stain is used to demonstrate the accumulation of lipid. (Appendix 4)

Result:

- Nuclei appear blue.
- Lipid appears red.[66]

3.3 Histological staining techniques

3.3.1 Perl's Prussian blue staining technique.

Haemosiderin is the protein compound by-product of degraded haemoglobin and is normally seen intracellularly as yellow to brown granules. Haemoglobin is insoluble in alkalis but soluble in acid solutions. Treatment with an acid ferrocyanide solution results in the unmasking of ferric iron as an hydroxide, $Fe(OH)_3$ by diluted hydrochloric acid. The ferric iron subsequently reacts with a diluted potassium ferrocyanide solution to produce an insoluble blue precipitate, ferric ferrocyanide. (Appendix 5)

Result: .Nuclei appears red. Haemosiderin appears blue.[66]

3.3.2 Periodic Acid-Schiff staining technique.

The Periodic acid-Schiff is one of the most widely used histological staining techniques used for the demonstration of carbohydrates and glycoconjugates. Carbohydrates contain free aldehyde groups which react with Schiff reagent to form a magenta-coloured compound. The intensity of the reaction depends to some extent on the length of the treatment in with the periodic and Schiff's solutions. (Appendix 6)

Results: Nuclei appear blue. Glycogens and glycoproteins appear magenta.[66]

3.4 Cytology differential counts and special stain quantitation.

Various cell counts were performed and recorded on a differential cell count and special stain quantitation form (Appendix 7). All cell counts were performed on the Olympus BX43 microscope using the 40x objective lens.

3.4.1 Differential cell counts

A differential count was performed on the white blood cells. 2 separate counts of 100 cells each were counted on the Papanicolaou and Rapidiff Giemsa stained slides.

Method:

1) The number of macrophages, neutrophils, lymphocytes and eosinophils were quantified using a white blood cell differential cell tally counter.
2) Two separate counts of 100 cells each were performed.
3) The average was taken and the result of each cell type was recorded as a percentage.

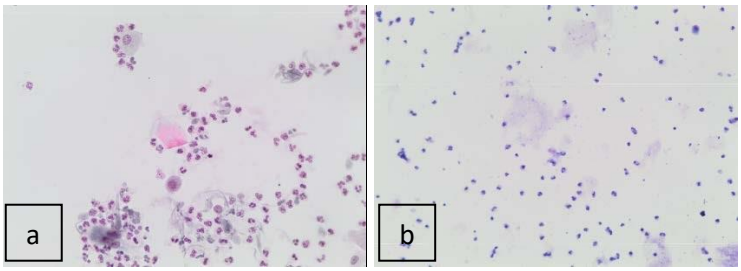


Figure 3.5: (a) Papanicolaou stained slide for differential cell counts. (b) Giemsa stained slide for differential cell counts.

3.4.2 Alveolar macrophage quantitation for lipid, haemosiderin and carbon.

Only macrophages larger than a granulocyte were counted on the same white blood cell counter. The first through to fourth knobs were used to count class I-IV alveolar macrophages respectively. Class III and IV carbon-laden macrophages were not expected, nor were they found.

Method:

1) The macrophages were classified as follows:
    - I: Negative for cytoplasmic lipid/haemosiderin/carbon black
    - II: approximately $^{1}/_{4}$ but less than $^{2}/_{4}$ of the macrophage contained phagocytosed lipid/haemosiderin/carbon black.
    - III: approximately $^{3}/_{4}$ of the macrophage contained phagocytosed lipid/haemosiderin/carbon black however negative spaces can still be seen.
    - IV: The cytoplasm is almost full or entirely full of lipid/haemosiderin/carbon black
2) Two counts of 100 macrophages were performed.
3) The average was taken of each class count and multiplied as follows to obtain a percentage (x-factor)
    - Class 1: Multiply by 0

- Class II: Multiply by 0.3
- Class III: Multiply by 0.6
- Class IV: Multiply by 1

4) The sum of each class x-factor was reported as a percentage.

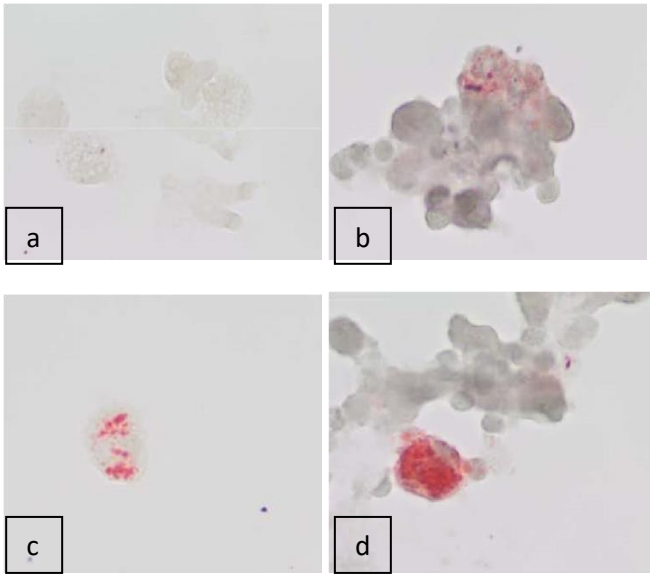


Figure 3.6: (a) Class I LLM. (b) Class II LLM. (c) Class III LLM. (d) Class IV LLM.

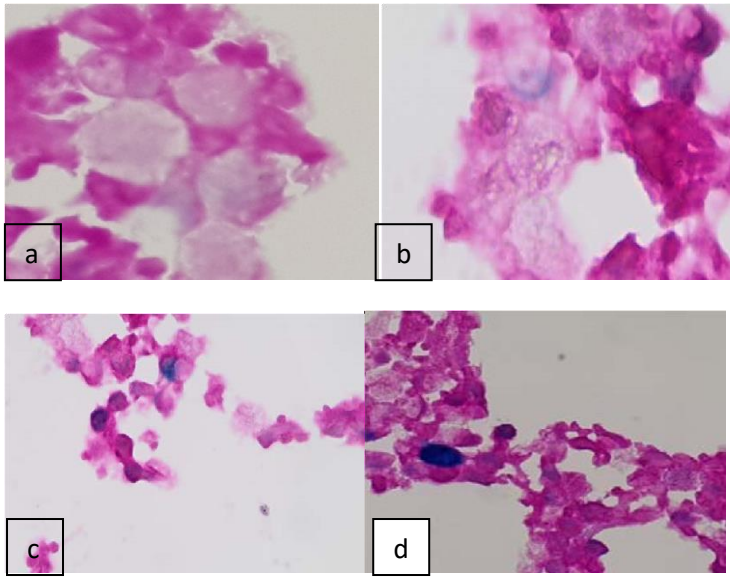


Figure 3.7: (a) Class I HLM. (b) Class II HLM. (c) Class III HLM. (d) Class IV HLM.

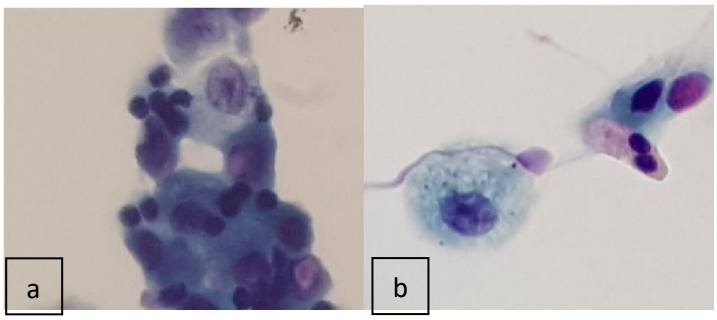


Figure 3.8 (a) Class I CLM. (b) Class II CLM.

3.4.3 Periodic acid-Schiff.

PAS positive macrophages were not quantified. If intracellular glycogen was seen, the PAS was either reported as positive or negative.

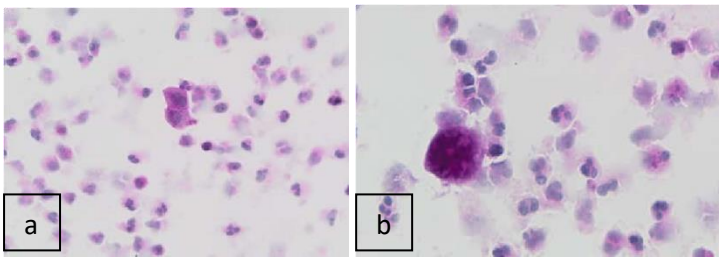


Figure 3.9: (a) PAS negative alveolar macrophage. (b) PAS positive alveolar macrophage.

3.5 Pulmonary tuberculosis investigations

3.5.1 GeneXpert.

GeneXpert® MTB/RIF is an automated, polymerase chain reaction cartridge based nucleic acid amplification assay used for the detection of *Mycobacterium tuberculosis* and rifampicin resistance by targeting the rBop gene of the mycobacterium.[67] (Appendix 8)

Result: If no growth is detected, a result of Negative for MTB complex will be given.

3.5.2 TB culture.

More acid-fast bacilli are required for visualization in direct microscopy in comparison to culture. Specimens sent for mycobacterial culture are either pulmonary in origin or extra-pulmonary and can be sorted into contaminated or non-contaminated. Contaminated samples, such as those of pulmonary origin undergo decontamination to eliminate bacteria other than mycobacteria with Sodium Hydroxde-N-Acetyl-L-Cysteine as per MGIT automation culture system recommendation. After processing, specimens are inoculated into a MGIT tube and entered into the BACTEC MGIT 960 System. A fluorescent compound embedded in the silicone on the bottom of the MGIT tube fluoresces when respiring micro-organisms consume oxygen. (Appendix 9)

Result: As per Tygerberg hospital, NHLS TB culture laboratory specific SOP, If no growth occurs, a result of "No growth after 40 days" will be given. If positive, the HAIN PCR is run as per manufacturer's instructions (Appendix 10).

3.5.3 Auramine O staining procedure and reporting.

Acid fast organisms emit a distinct bright yellow fluorescence with the potassium permanganate counter stain. Positive fluorescent acid-fast bacilli are seen slightly curved rod-shaped micro-organisms. (Appendix 11)

3.6 Microbiological investigations

3.6.1 Bronchoalveolar lavage fluid gram stain

The gram stain is used to classify bacteria based on their size; shape and cellular morphology. Bacteria stain either Gram-positive or gram negative based on architecture and composition of their cell wall. Gram-positive bacteria have a thick peptidoglycan layer and thus retain their initial deep violet stain as they are unaffected by alcohol decolourisation. Gram-negative bacteria have a single peptidoglycan layer and is decolourised by alcohol, allowing the microorganism to be stained by counterstains. (Appendix 12)

Results: Gram positive micro-organisms remain violet, gram-negative micro-organisms are pink.

3.6.2 Bronchoalveolar lavage fluid microbiology culture.

Microbiological culture of sputum samples are ineffective at determining whether a pathogen is causing a lower or upper respiratory tract infection [70]. A gram stain is performed on BALF to detect the presence of active inflammation in the lower respiratory tract as well as assist in determining the most likely organism. Specimen is used to inoculate various culture plates.[68] (Appendix 13)

3.7 Chemical pathology investigations

3.7.1 Sweat test.

Chloride is one of the sweat electrolytes increased in patients suffering from cystic fibrosis. Pilocarpine is a drug that stimulates the sweat glands to enable for an adequate sweat sample collection. Iontophoresis is performed whereby positively charged pilocarpine ions are moved into the skin between 2 electrodes whilst current is being passed. The sweat is collected on pre-weighted filter paper and extracted (Appendix 14). Extracted chloride is then analyzed as per manufacturer's instructions (Appendix 15). This test can be performed on normally hydrated infants, 2 weeks of age and weighing more than 3kgs without significant systemic illness according to NHLS Tygerberg chemical pathology laboratory subject suitability..

Results:

- <40mmol/l: Cystic fibrosis is unlikely
- 40-59mmol/l: Suggestive of cystic fibrosis but not diagnostic.
- >59mmol/l: Supports the diagnosis of cystic fibrosis

3.7.2 Serum Ig A.

Ig A is the predominant immunoglobulin in bodily secretions such as sweat and saliva. It serves to protect the skin and mucosa against micro-organisms by binding to toxins and in conjunction with lysozyme, results in anti-bacterial and antiviral activity. Increased IgA levels are seen in chronic infections and sarcoidosis, whilst decreased IgA levels are seen in acquired and congenital immunodeficiency diseases. IgA assays are used to determine serum and plasma IgA levels.

The IgA assay is an immunoturbimetric assay in which anti-IgA antibodies react with the IgA antigen in the sample to agglutinate and form an antigen-antibody complex. The aggregates are determined turbidimetrically. (Appendix 16)

3.7.3 Serum Ig G.

IgG is a maternal immunoglobulin that can cross the placental barrier and provide passive immune protection to a foetus. The main role of IgG is to provide defence against microorganisms by directly neutralizing a toxin via an induction of complement fixation. IgG level gradually decline as the infant's own immunological system develops. Increased IgG levels are seen in infectious diseases and cystic fibrosis.

The IgG assay is an immunoturbimetric assay in which anti-IgG antibodies react with the IgG antigen in the sample to agglutinate and form an antigen-antibody complex. The aggregates are determined turbidimetrically. (Appendix 17)

3.7.4 Serum Ig M.

IgM is the first immunoglobulin to appear in the serum after an infection. It aids in the killing of bacteria by activating complement. Unlike IgG, the IgM serum levels decrease rapidly once the infection has cleared and allows for the differential diagnosis when comparing the two antibodies. Increased IgM levels are found in infections (viral; bacterial and parasitic), cystic fibrosis and heroin addiction. IgM levels are generally lower in infants due to the slow onset of IgM synthesis.

The IgM assay is an immunoturbimetric assay in which anti-IgM antibodies react with the IgM antigen in the sample to agglutinate and form an antigen-antibody complex. The aggregates are determined turbidimetrically. (Appendix 18)

3.7.5 C-Reactive Protein.

C-reactive protein is an acute phase protein found in inflammatory reactions and is synthesized by the liver. CRP assays are used to detect systemic inflammatory responses. The test involves a particle enhanced immunoturbimetric assay where human CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies. The aggregates are determined turbidimetrically. (Appendix 19)

3.8 Virology investigations

The basic principle of polymerase chain reaction (PCR) is that one DNA molecule is used to produce exponential copies. DNA is made up of 4 nitrogenous bases, namely adenine (A), thymine (T), cytosine (C) and guanine (G). This continuous doubling is achieved by using specific proteins called polymerases, which are enzymes that can string together individual DNA bases to form long strands. These polymerases require a primer which is DNA fragments that bind to the bases. A target DNA molecule that serves as a template.

3.8.1 Respiratory virus multiplex

The Anyplex® II RV16 PCR system is a qualitative multiplex real-time polymerase chain reaction system using TOCE™ technology which allows for the detection of multiple pathogens from respiratory tract (nasopharyngeal aspirate, nasopharyngeal swab and bronchoalveolar lavage) specimens in a single fluorescence channel on real-time PCR instruments. Multiple amplifications of target RNA/DNA for 16 viruses in 2 panels can therefore be performed. The viruses detected are Influenza A virus, Influenza B virus, Human respiratory syncytial virus A, Human respiratory syncytial virus B, Human adenovirus, Human metapneumo virus, Human coronavirus 229E, human coronavirus NL63, Human coronavirus OC43, Human parainfluenza virus 2, Human parainfluenza virus 3, Human

parainfluenza virus 4, Human rhinovirus A/B/C, Human enterovirus and Human bocavirus 1/2/3/4. (Appendix 20)

3.8.2 Cytomegalovirus

Cytomegalovirus (CMV) is a DNA virus from the family *Herpesviridae*. It affects humans of all ages and has no seasonal or epidemic patterns of transmission. The bulk of CMV infections are asymptomatic, however may become a life-threatening disseminated disease in immunocompromised patients. (Appendix 21**)**

Chapter 4: Results

4.1 Meanage in months.

The study has a total of 90 patients in the category of "chronic wheeze". 23 Samples were rejected due to poor cellularity and age exclusion. Of the 67 patients with BALF adequate for evaluation, 27 were female and 40 were male. Of these 67 patients, the mean age was 20.88 months. The youngest patient, both male and female, was 3 months of age and eldest was 60 months old.

The broad category final outcome diagnosis of infection/ inflammation (n=36) had a mean age of 22.92 months compared to the recurrent wheezer (n=31) who were slightly younger and had a mean age of 18.52 months.

When comparing the sub-categories, older children with a mean age of 24.95 months were found to have infection/inflammation (n=23), compared to recurrent wheeze (n=13) who had a mean age of 18.45 months; GORD (n=9) had a mean age of 15.54 months; structural abnormalities (n=14) had a mean age of 14.57 months; IPF (n=1) was 13 months and alveolar proteinosis (n=1) was 14 months. Six were initially lost to follow-up but later were placed in the broad category of recurrent wheeze and recurrent infection.

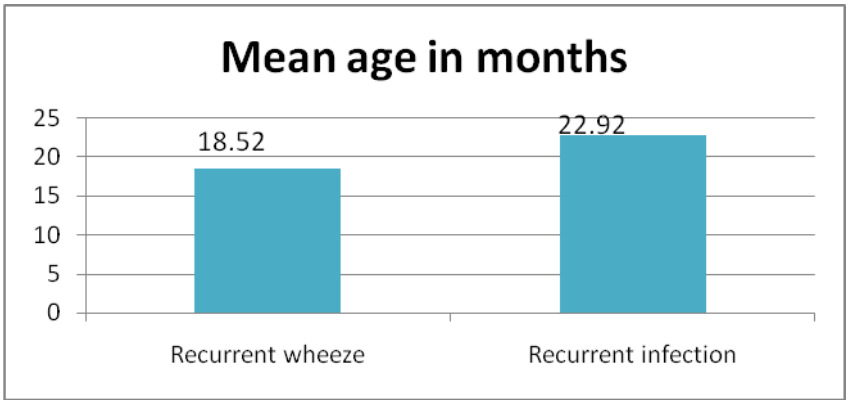


Figure 4.1: Main category age in months comparison.

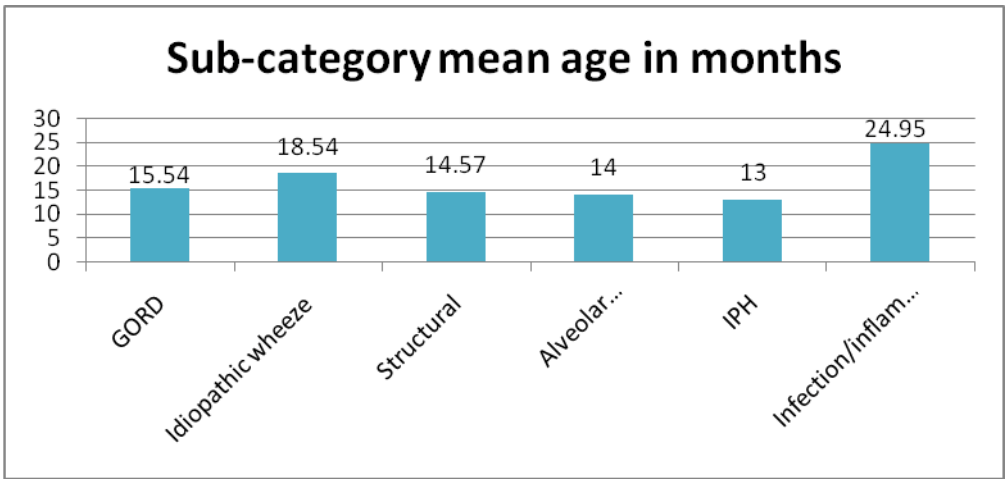


Figure 4.2: Sub-category age in months comparison.

4.2 Cytology cell count results.

4.2.1 Differential cell count results.

The 2 isolated cases of alveolar proteinosis and idiopathic pulmonary haemosiderosis were added to the inflammation/infection category the sub-category differential counts.

The 2 sample t-test with equal variances, also known as a mean comparison test, was applied to see if the differential cell count means of the 2 populations of "recurrent infection" vs "recurrent wheeze" were equal.

The results of the compared means for the different cell types in the BALF for the 2 broad categories were as follows:

Table 4.1: Main category comparison of differential cell counts.

Category	Mean Macrophage %	Mean Neutrophil %	Mean Lymphocyte %	Mean Eosinophil %
Recurrent infection	27	60	12	0.7
Recurrent wheeze	32	53	13	0.8
Significance	Pr(|T| > |t|) = 0.3600	Pr(|T| > |t|) = 0.3426	Pr(|T| > |t|) = 0. 7852	Pr(|T| > |t|) = 0.8353

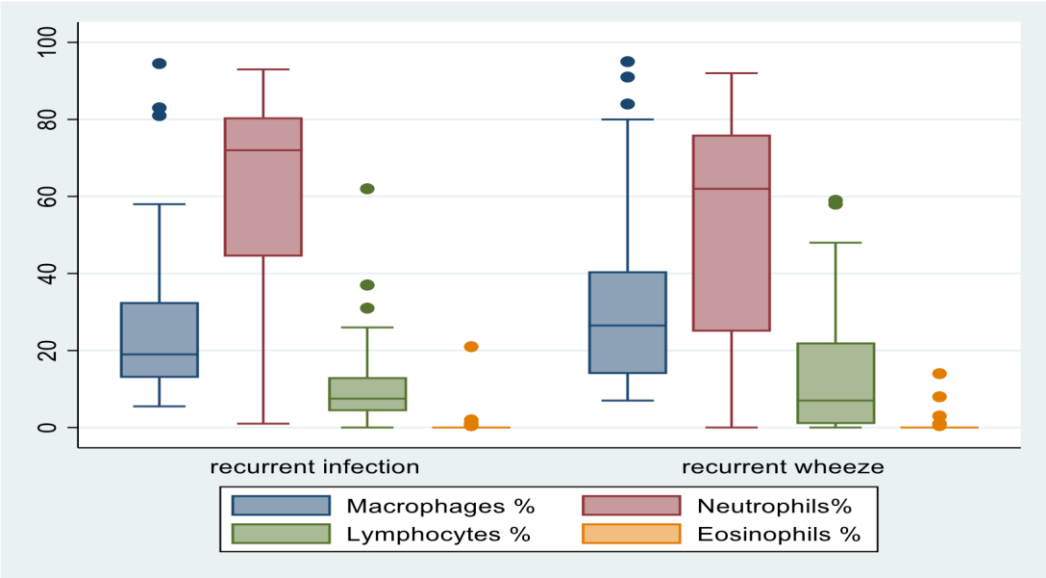


Figure 4.3: Main category differential cell count comparison.

There was a fairly even split when comparing the mean differential cell counts of the recurrent infection and recurrent wheeze. The same applied to the sub-category comparison.

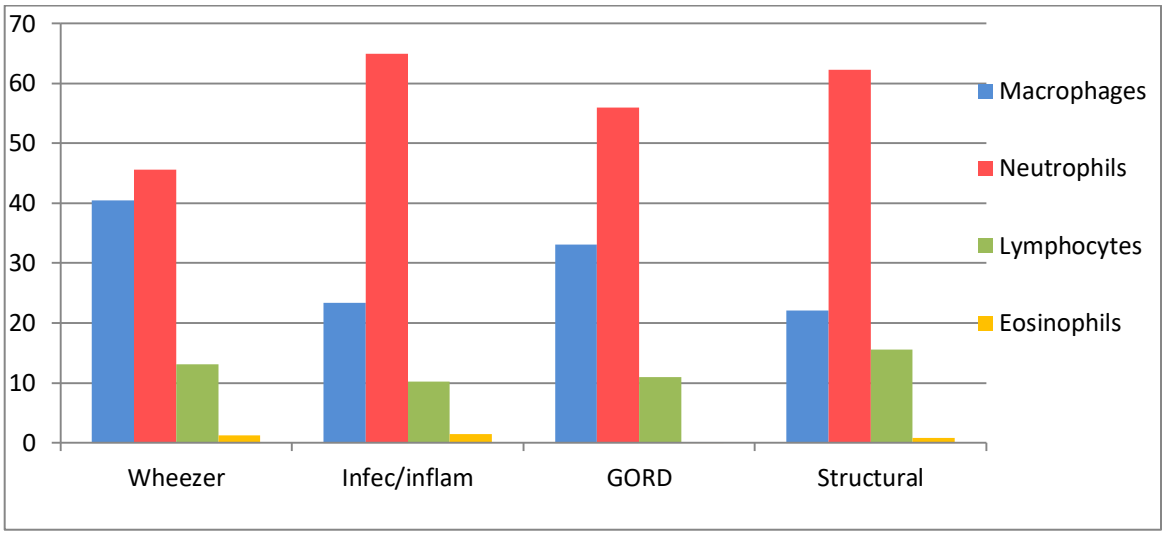


Figure 4.4: Sub-category differential cell counts comparisons.

When comparing the broad categories, recurrent wheeze had a mean macrophage score of 32% compared to recurrent infection which had a mean macrophage score of 27%. The mean neutrophil score in the recurrent wheeze was 53% compared to the mean neutrophil score of

60% in recurrent infection. There was a 1% difference in the lymphocyte score with recurrent wheeze having a mean lymphocyte score of 13% vs that of the 12% mean lymphocyte score seen in recurrent infection. Similarly, the mean eosinophil score was 0.8% in recurrent wheeze and 0.7% in recurrent infection. There was no statistically significant difference between the means of the macrophages, neutrophils, lymphocytes or eosinophils of the 2 broad groups of recurrent infection vs recurrent wheezer. When comparing the differential cell count cell profiles between the various sub categories, the chronic wheezer had the highest mean macrophage score of 40.46% followed by GORD which had a mean macrophage score of 33.07%. The structural abnormality and infection/inflammation sub-category shared a very similar lower mean macrophage score with structural abnormality scoring 22.12% and infection/inflammation scoring 23.37%. There was no statistically significant difference between the means of the macrophages, neutrophils, lymphocytes and eosinophils when comparing the sub-categories against each other.

4.2.2 Carbon, lipid and haemosiderin-laden macrophage results.

A median regression and 2 sample t-test with equal variances was performed on the 2 main groups comparing the mean carbon, lipid and haemosiderin counts of the 2 groups.

Table 4.2: Main category comparison of carbon, oil red O and haemosiderin variables.

Category	**Mean carbon-laden macrophage %**	**Mean lipid-laden macrophage %**	**Mean haemosiderin laden macrophage %**
Recurrent infection	4.718571	8.499444	8.900571
Recurrent wheeze	6.258064	6.496774	5.883226
Significance	Pr(|T| > |t|) = 0.1523	Pr(|T| > |t|) = 0.4756	Pr(|T| > |t|) = 0.4215

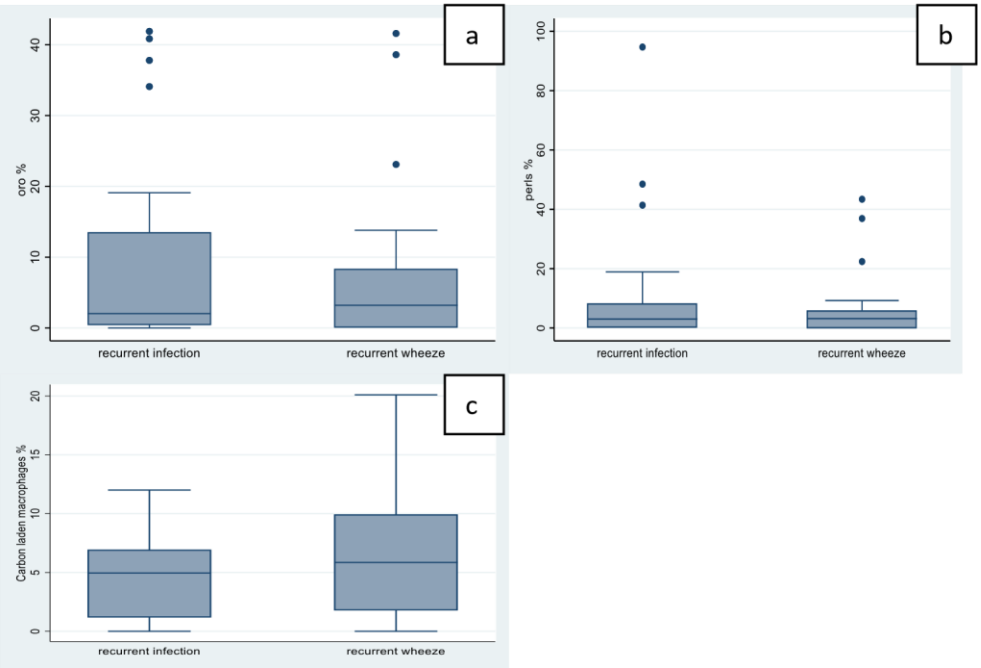


Figure 4. 5: (a) Main category comparison of ORO variable. (b) Main category comparison of Perl's variable. (c) Main category comparison of carbon variable.

There was no statistically significant difference between the means of the lipid-laden, haemosiderin-laden or carbon laden macrophages of the 2 broad groups of recurrent infection vs. recurrent wheeze.

Next, the sub-groups were compared.

Table 4.3: Sub-category comparison of carbon ORO and haemosiderin variables.

Category	Mean carbon-laden macrophage %	Mean lipid-laden macrophage %	Mean haemosiderin laden macrophage %
GORD	6.605556	3.094444	2.727778
Idiopathic wheeze	7.984615	7.288461	9.040769
Infection/ inflammation	4.780435	12.66652	4.630435
Structural abnormality	3.557143	5.721429	10.52846

Single values as with IPF and alveolar proteinosis are indicated as a single line due to only one case of each being present. Only 1 patient had a final outcome of alveolar proteinosis after the full clinical work-up.

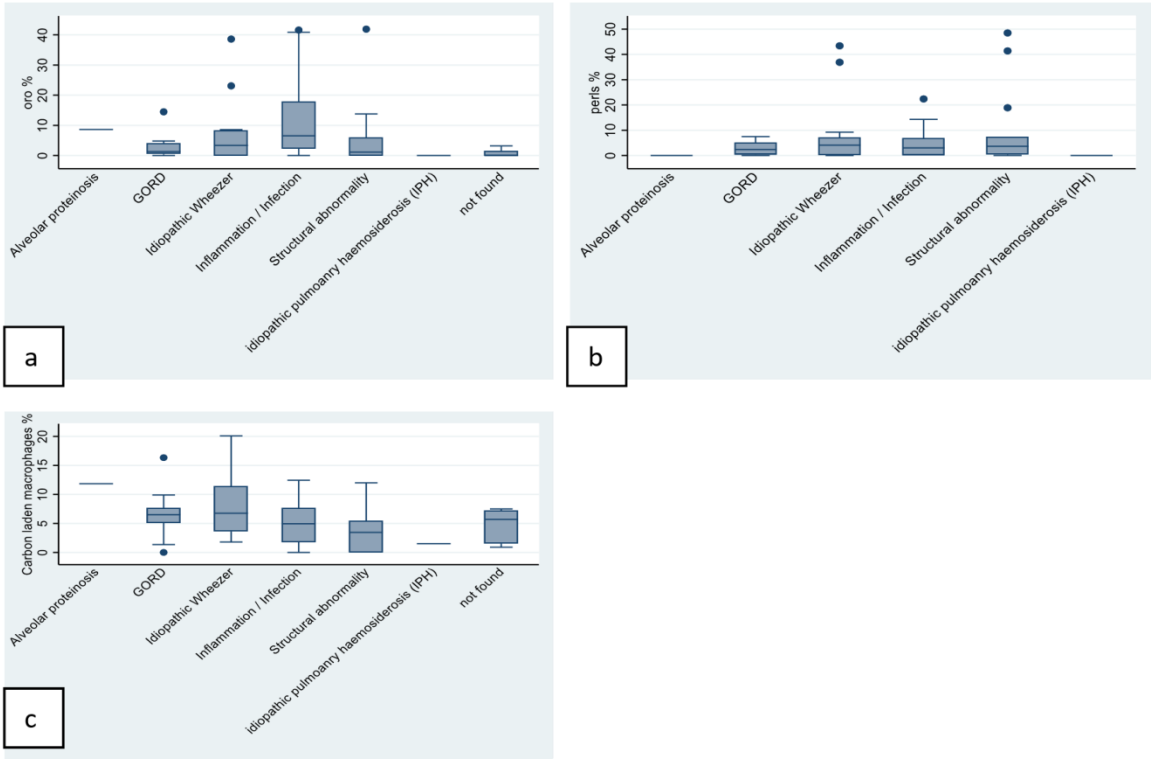


Figure 4.6: (a) Sub-category comparison of ORO variable. (b) Sub-category comparison of Perl's variable. (c) Sub-category comparison of carbon variable.

GORD was used as the reference as it showed the least amount of both lipid-laden and haemosiderin-laden macrophages.

The infection/inflammation sub-group showed the highest number of lipid-laden macrophages with a p50 value of 6.55. The idiopathic wheezer sub-group showed the highest number of carbon-laden macrophages and haemosiderin-laden macrophages.

When comparing the other 3 categories, using GORD as the constant. Carbon had the highest value in the wheezer category and overall, it was not considered to be significant.

Table 4.4: Sub-category comparison of carbon, lipid and haemosiderin against a constant (GORD).

Category	**Mean carbon-laden macrophage %**	**Mean lipid-laden macrophage %**	**Mean haemosiderin laden macrophage %**
Idiopathic	2.779167	3.835762	2.345917

wheeze			
Infection/ inflammation	2.484722	3.473902	2.128534
Structural abnormality	0.3291669	3.169685	1.942519
Age in months	.0680556	.085515	.0517723
Cons(GORD)	2.0875	2.703297	1.696885
Significance	**Prob > F = 0.4382**	**Prob > F = 0.2848**	**Prob > F = 0.1126**

Age adjustment allowed for the distribution ages of the patients in the study to be compared as variables. In the case of haemosiderin-laden macrophages, it revealed that GORD patients were younger (<20months) and had a lower haemosiderin-laden macrophage index than that of the infection/inflammation sub-category who had a wider age range and a higher haemosiderin-laden macrophage index. This is seen in figure 4.5.

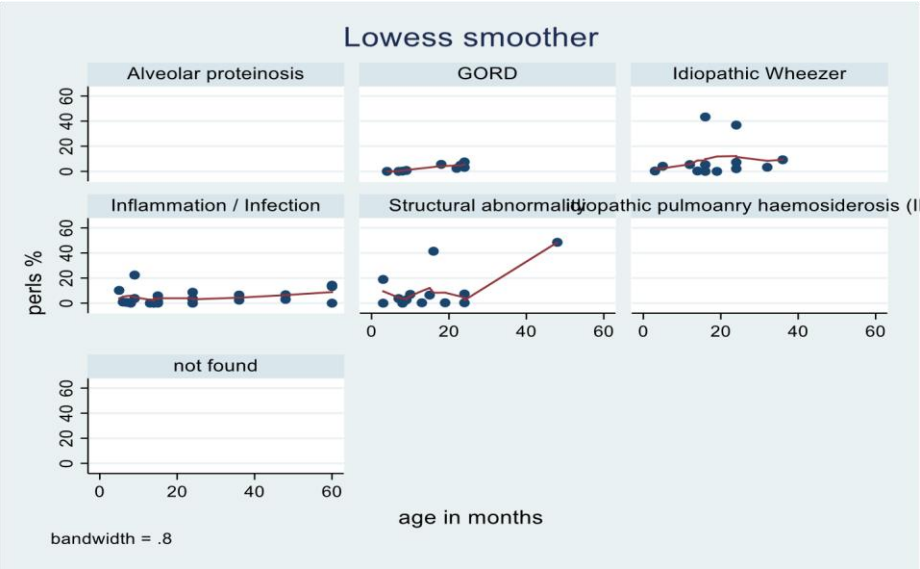


Figure 4.7: Age adjustment comparison of sub-category to Perl's variable.

4.2.3 Periodic acid-schiff results.

The single case of alveolar proteinosis was PAS positive. A total number of 49 PAS positive results were reported in this study.

4.3 Pulmonary tuberculosis investigations results.

There were a total of 2 cases with trace and intermediate MTB complex detected, these however all had a negative culture and Auramine O result.
Barring the 2 aforementioned results, the following results were reported on the BALF of the patients involved in this study:

- TB culture: Negative (no growth after 40 days).
- GeneXpert® MTB/RIF: Negative MTB complex not detected.
- Auramine O: Negative.

4.4 Microbiology investigations results.

4.4.1 Gram stain results

In the 2 broad category groups', gram stain results were compared against one another, it was found they shared very similar results, however, the recurrent wheezer had a slightly higher number of neutrophils with no bacteria observed on their BALF gram stain with no growth after 2 days.

7 out of the 9 cases of the recurrent wheezer revealed a negative gram stain result consisting of neutrophils and no bacteria and no bacterial growthseen on the culture of their BALF.

Table 4.5: Main category recurrent wheezer and recurrent infection gram stain comparison.

Category	Negative gram stain	Neutrophils, no bacteria	Gram + cocci	Gram - bacilli
Wheezer	9 (n)	13 (n)	6 (n)	3(n)
Infection/ inflammation	10 (n)	11 (n)	6 (n)	3 (n)

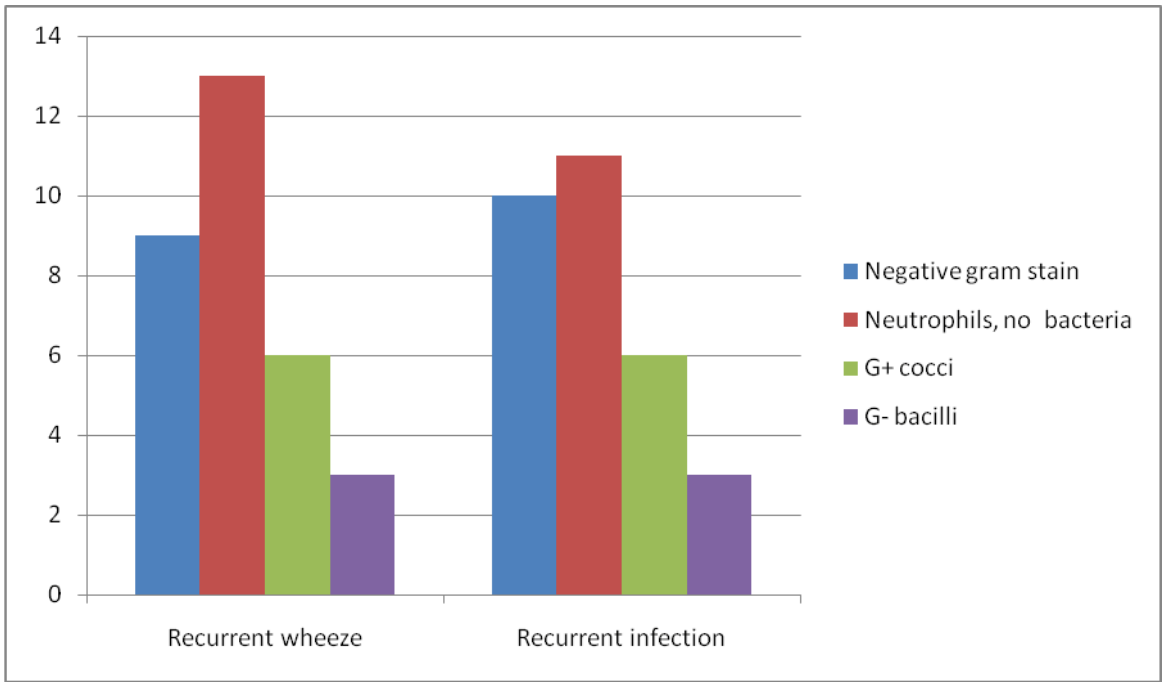


Figure 4.8: Microbiology gram stain results comparison between recurrent wheezer and recurrent infection.

4.4.2 Culture results

When BALF culture results were compared against one another in the 2 broad category groups', they shared very similar results. A greater variety of species were cultured from the BALF of the recurrent infection category than that of the recurrent wheezer category.

Table 4.6: Main category recurrent wheezer and recurrent infection BALF microbiology culture sample outcome comparison.

Category	No growth	Normal flora	Positive culture	Not requested
Wheezer	(n)16	(n)5	(n)8	(n)2
Infection/ inflammation	(n)15	(n)6	(n)7	(n)7

Table 4.7: Main category recurrent wheezer and recurrent infection BALF microbiology cultured species comparison.

Recurrent wheezer	Recurrent infection
• Normal flora isolated (5) • H. Influenza (4) • Klebsiella pneumoniae(1) • Streptococcus pneumonia (4)	• Normal flora isolated (7) • H. influenza(4) • M.Catarrhalis (1) • Klebsiella oxytoca (1) • Streptococcus pneumonia (1) • Mixed growth observed (1)

4.5 Chemical pathologyinvestigation results.

4.5.1 Sweat test.

Out of 43 sweat tests requested in total, 39 were negative and indicated that cystic fibrosis was unlikely. There were 4 reports that had an intermediate report for cystic fibrosis indicating that cystic fibrosis may be possible. 2 were from the recurrent wheeze category and 2 were from the recurrent infection category. There was no final diagnosis of cystic fibrosis those 4 cases.

4.5.2 IgM.

The IgM variable was initially compared in the 2 broad categories of chronic wheezer and recurrent infection. The IgM in the recurrent infection category was slightly higher than that of the recurrent wheeze category; however it was not statistically significant.

When the IgM variable was then compared in the sub-categories, it was highest in the idiopathic wheezers and more normal in the inflammation, structural abnormality and GORD categories. This however was not statistically significant.

Table 4.8: Main category IgM comparison.

Category	Hi IgM	Not requested	Normal IgM
Recurrent infection	(n)6 16.67 %	(n)14 38.89 %	(n)16 70.83 %
Recurrent wheeze	(n)3 9.68 %	(n)10 32.26 %	(n)18 58.06 %
Significance	Pearson chi2(2) = 1.4191 Pr = 0.492		

Table 4.9: Sub-category IgM comparison.

Category	Hi IgM	Not requested	Normal
Alveolar proteinosis	(n)0 0.00 %	(n)0 0.00 %	(n)1 100.00 %
GORD	(n)0 0.00 %	(n)4 44.44	(n)5 55.56 %
Wheezer	(n)3 23.08 %	(n)5 38.46	(n)5 38.46 %
Infection/ inflammation	(n)3 13.04%	(n)6 26.09	(n)14 60.87%
Structural abnormality	(n)2 14.29%	(n)5 35.71%	(n)7 50.00%
IPH	(n)0 0.00%	1 100.00	(n)0 0.00%
Not found	(n)1 16.67%	(n)3 50.00 %	(n)2 33.33 %
Significance	Fisher's exact =0.838		

4.5.3 IgG.

The IgG variable was compared between the broad category chronic wheezer and recurrent infection. The IgG in the recurrent infection category almost double that of the recurrent wheeze category, however it was not statistically significant.

When the IgG variable was then compared in the sub-categories, it was similarly elevated in the infection/inflammation; wheezer and structural abnormality categories, with a higher normal distribution in GORD. This however was not statistically significant.

Table 4.10: Main category IgG comparison.

Category	Hi IgG	Low IgG	Normal IgG
Recurrent infection	(n)13 59.09 %	(n)1 4.55 %	(n)8 36.36 %
Recurrent wheeze	(n)7 29.17 %	(n)0. 0.00 %	(n)17 70.83%
Significance	Fisher's exact = 0.037		

Table 4.11: Sub-category IgG comparison.

Category	Hi IgG	Low IgG	Not requested	Normal
Alveolar proteinosis	(n)1100.00 %	(n)0. 0.00 %	(n)0 0.00 %	(n)0 0.00%
GORD	(n)0 0.00%	(n)0 0.00 %	(n)3 33.33%	(n)6 66.67 %
Wheezer	(n)5 38.46%	(n)0 0.00 %	(n)4 30.77%	(n)4 30.77%
Infection/ inflammation	(n)8 34.78 %	(n)1 4.35%	(n)6 26.09%	(n)8 34.78%
Structural abnormality	(n)5 35.71 %	(n)0 0.00 %	(n)4 28.57%	(n)5 35.17%
IPH	(n)0 0.00 %	(n)0 0.00 %	(n)1 100.00	(n)0 0.00
Not found	(n)1 16.67 %	(n)0 0.00 %	(n)3 50.00 %	(n)2 33.33 %
Significance	Fisher's exact =0.615			

4.5.4 IgA.

The IgA variable was compared between the broad category chronic wheezer and recurrent infection. Although the IgA distribution was higher in the recurrent infection when compared to the recurrent wheeze category, it was not statistically significant.

The IgA variable was fairly similar when compared between the various sub-categories. No statistical significance was found.

Table 4.12: Main category and sub-category IgA comparison.

Category	Hi IgA	Low IgA	Not requested	Normal IgA
Recurrent infection	(n)5 13.89%	(n)1 2.78 %	(n)14 38.89 %	(n)16 44.44%
Recurrent wheeze	(n)3 9.68 %	0. 0.00%	(n)7 22.58 %	(n)21 67.74%
Significance	Pearson chi2(3) = 4.1590 Pr = 0.245			

Table 4.13: Sub-category IgA comparison.

Category	Hi IgA	Low IgA	Not requested	Normal
Alveolar proteinosis	(n)0 0.00 %	(n)0. 0.00 %	(n)0 0.00%	(n)1 100.00 %
GORD	(n)1 11.11 %	(n)0 0.00 %	(n)3 33.33%	(n)5 55.66 %
Wheezer	(n)1 7.69 %	(n)0 0.00 %	(n)4 30.77 %	(n)8 61.54 %
Infection/ inflammation	(n)3 13.04 %	(n)1 4.35 %	(n)6 26.09 %	(n)13 56.52%
Structural abnormality	(n)2 14.29 %	(n)0 0.00 %	(n)4 28.57 %	(n)8 57.14 %
IPH	(n)0 0.00 %	(n)0 0.00 %	(n)1 100.00 %	(n)0 0.00 %
Not found	(n)1 16.67 %	(n)0 0.00 %	(n)3 50.00 %	(n)2 33.33%
Significance	Fisher's exact =0.988			

4.5.5 C-reactive protein (CRP).

When comparing the C-reactive protein variable against the various sub-categories, it was highest in the inflammation and infection category and fairly evenly distributed amongst the wheezer, GORD and structural sub-categories. This however, was not statistically significant.

Table 4.14: Sub-category C-reactive protein comparison.

Category	Hi CRP	Not requested	Normal
Alveolar proteinosis	(n)0 0.00%	(n)0 0.00%	(n)1 100.00 %
GORD	(n)6 66.67%	(n)1 11.11 %	(n)2 22.22%
Wheezer	(n)5 38.46 %	(n)5 38.46 %	(n)3 22.08 %
Infection/ inflammation	(n)12 52.17 %	(n)8 34.74 %	(n)3 13.04 %
Structural abnormality	(n)6 42.89%	(n)5 35.71 %	(n)3 21.43%
IPH	(n)1 100.00 %	(n)0 0.00%	(n)0 0.00 %
Not found	(n)3 50.00 %	(n)2 31.34 %	(n)13 19.40 %
Significance	Fisher's exact = 0.855		

4.6 Virology investigations

4.6.1 Respiratory virus multiplex comparison outcome between recurrent wheeze vs. infection.

When the 2 broad category groups' virology results were compared against one another, it was found there was more viral species isolated in the recurrent wheezer category than in the recurrent infection category.

Table 4.15: Broad category wheezer virology respiratory virus multiplex comparison.

Recurrent wheezer	Recurrent infection
• Adenovirus (11) • Coronavirus OC43 (3) • Coronavirus OC63 (1) • Enterovirus (4) • Human rhinovirus (17) • Human metapneumovirus (1) • Human bocavirus (1) • Influenza A (1) • Parainfluenza 3 (1) • Respiratory scyntial A (4)	• Adenovirus, (11) • Enterovirus (7) • Influenza B (1) • Human rhinovirus (18) • Human bocavirus (2) • Parainfluenza 1(2) • P parainfluenza 3 (1) • Respiratory scyntial A (1)

4.6.2 CMV comparison outcome between recurrent wheeze vs. infection.

A total of58 patients were tested for CMV in this study. Of these, 26 were from the chronic wheezer category and 32 were from the recurrent infection category.

7 Patients in the recurrent wheezer category tested negative and 19 tested positive. Out of the recurrent infection category, 10 tested negative and 22 tested positive.

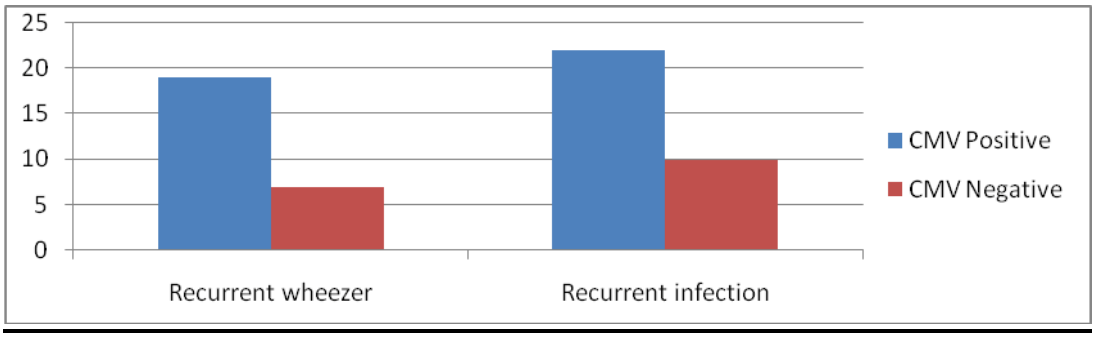


Figure 4.9: CMV virology results comparison between recurrent wheezer and recurrent infection.

Chapter 5: Discussion.

5.1. Discussion.

In normal individuals 80-90% of cells recovered from BALF are macrophages, followed by 5-15% lymphocytes and 3% neutrophils and 1% eosinophils respectively. This BALF cellular profile is similar in paediatric patients. If a patient presents with no obvious diagnosis, formulating a final diagnosis on very non-specific symptoms is often results in a diagnosis of exclusion being made. Performing cytological analysis on paediatric BALF may be useful in the determination of the cellular constituents within the BALF thus ruling out certain pulmonary conditions.

This study involved 90 paediatric patients with the clinical presentation of a recurrent wheeze with or without an accompanying recurrent infection.

When comparing the mean ages, the broad categories of recurrent wheezer vs recurrent infection, their age mean differed by approximately 4 months, with the recurrent wheezers being younger in age. When looking at the mean ages of the sub-categories, it was seen that GORD patients were primarily younger (15.54 months) compared to the infection/inflammation category(24.95 months).

The differential cell counts, where very similar across the categories and revealed no statistical significance between the broad category of recurrent wheezer vs. recurrent inflammation. The differential cell counts revealed that if compared to the “norm” of 80% macrophages, their counts were greatly decreased and their neutrophil count was almost 18 times greater than what is considered to be normal.

When comparing the carbon, lipid and haemosiderin-laden macrophages of the 2 broad categories, although not statistically significant, the analysis revealed that there was a greater percentage of carbon-laden macrophages in the recurrent wheezer (6.258%) compared to the recurrent infection category(4.718%), whereas the recurrent infection category had higher lipid-laden macrophage percentage (8.499%)and haemosiderin-laden macrophage percentage (8.900%) compared to percentages seen in the chronic wheezer category for lipid-laden macrophages (6.496%) and haemosiderin laden macrophages(5.888%).

A potential source of inaccuracy pertaining to cell quantitation may be due to inconsistency during interpretation of the measuring tools or variables which are subjective; these variables include cellular quantitation of leukocytes and alveolar macrophages, cellularity of the cytospin, quality of the stain the amount of phagocytosed material. Interobserver variability and the aforementioned parameters may result in quantitation and grading disparities.[55,57,58]

Although not statistically significant, the statistical analysis revealed that the subcategory comparison revealed chronic wheezers have a higher mean carbon-laden macrophage percentage (2.27795%) compared to infection/inflammation (2.4845%); structural abnormality(0.3291%) and GORD(2.08%) sub-categories. The comparison also revealed that GORD patients are both younger in their mean age and have the lowest mean haemosiderin and lipid-laden macrophage score indicating that not all GORD patients aspirate and thus BALF in paediatric GORD patients can assist in excluding the diagnosis of GORD pulmonary aspiration disease.

All of the immunoglobulins were elevated in the recurrent infection category compared to recurrent wheezers. When the subcategories were compared, there was a fairly even distribution but IgA in the recurrent wheezer category was slightly decreased (7.69%) compared to the other sub-categories which revealed 13.04% in the infection/inflammation subcategory and 14.29% in the structural abnormality subcategory.. The CRP analysis in the sub-groups revealed GORD as having the highest elevated serum CRP parameter.

Barring the minority of patients who had a positive outcome, the TB and cystic fibrosis investigations were negative in the patients involved in this study

The microbiological investigations in the study were similar in the 2 broad category groups, however it revealed that a greater variety of cultured micro-organisms were found in the recurrent infection group. The opposite was found in the respiratory virus virology BALF investigations whereby a larger variety of viruses were isolated in the BALF of recurrent wheezers when compared to recurrent infection.

In this study, the overall agreement was that none of the tests were of statistical significance used in the investigation of paediatric chronic wheezers were significant for the formulation of a specific diagnosis, or ability to place this group of children into a specific diagnostic category. The low sample size and lack of a control group are the main limitations of this

study. There is adequate room to elaborate on this study by barring the exclusion criteria and performing statistical analysis on all paediatric BALF received at Tygerberg hospital by the paediatric pulmonology unit; collaborating with a private cytology laboratory that processes BALF to ascertain the difference between carbon-laden macrophages of children in a private vs government setting; explore automation as a potential method for cellular quantitation in our setting as well as including a test for pepsin on GORD patients. In this way hopefully decrease the time spent on processing the samples and interobserver variability, truly differentiate between aspirated gastric content and aspirated lipid as culturally some mothers feed their babies oil in our setting. Further studies into fine-tuning the applications of BALF can decrease the turn-around time for the test and improving the accuracy as well as provide valuable information to the clinician and subsequently patient.

Chapter 6: Conclusion.

6.1 Conclusion

The primary aim of paediatric BALF specimen collection and analysis from the pulmonology unit at Tygerberg hospital is to assist in the diagnosis of GORD aspiration pulmonary disease, an infective pathology, specific diagnosis[2] such as alveolar proteinosis, atopy related hypersensitivity conditions or to assist in a diagnosis of exclusion.

Although not necessarily a diagnostic test, the cytological analysis of paediatric BALF should not be disregarded. The time-consuming nature of the test may be improved if the option of electronic cell counts were to be performed. In addition, this test may be of more benefit to the clinician and subsequently the patient if more accurate investigations such as pepsin and α-lactalbumin immunocytochemical stains were to be utilized. This however is not current practice in our laboratory due to a lack of resources, Should future studies reveal that this aids the clinician in their final diagnosis then this will be added to our panel of tests performed on BALF.

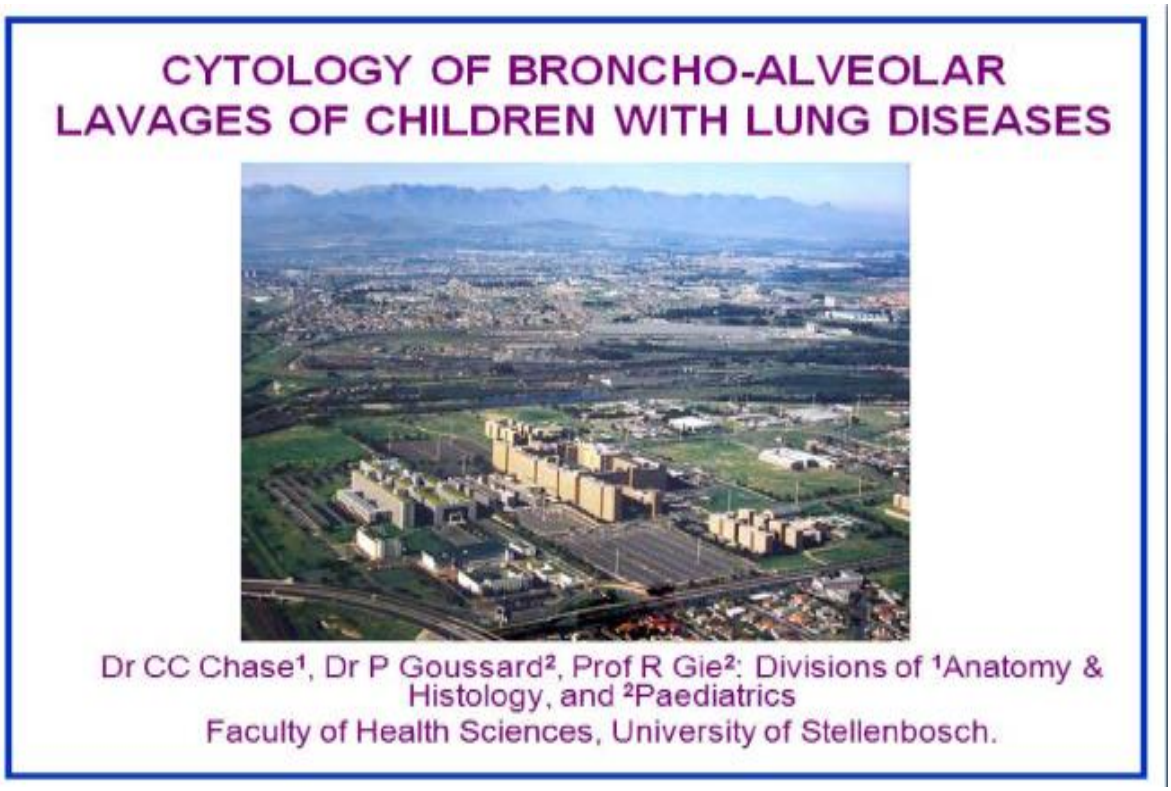
With the global increase in interest in environmental air pollution and emerging studies involving pulmonary disease development and exacerbation in relation to inhaled particulate matter; and its adverse effect on paediatric patients (in light of their behavioural and physiological factors), including a carbon-laden macrophage index and correlating it to a larger sample size not confined to a category of recurrent wheeze may provide much needed information that can only be obtained from a third world setting. Further investigations involving the size and composition of particulate matter and carbon loading of the alveolar macrophages in a paediatric group with a clinical correlation may also add to value to the current literature and bring global awareness to a seemingly innocuous ambient air constituents.

This study has demonstrated that routine cytology processing of paediatric BALF that were obtained via a minimally invasive, BAL procedure performed by skilled pulmonologists could assist in the exclusion of various pulmonary diseases related to a chronic wheeze and recurrent pulmonary infections.

The cytology differential cell count and grading of carbon, lipid and haemosiderin as well as clinical pathology and virology investigations did not reveal any significant results with regards to placing these children into a sub-classification or determining if the amount of phagocytosed carbon corresponds to an increased risk of developing chronic lung injury. Additional studies with a much larger sample size and correlation to a "normal" group are needed.

Appendix 1(a): Dr Chase BALF processing and cell count method.

This method was presented at the 37th Annual Conference of the Anatomical Society of Southern Africa, 2007.22-25 April.



Broncho-alveolar lavage cytology in children with lung disease. By C.C. CHASE[1], R GIE[2], P GOUSSARD[2], *Divisions of [1]Anatomy and Histology and [2]Paediatrics, Faculty of Medicine, University of Stellenbosch, P.O. Box 19063, Tygerberg 7505.* ccc@sun.ac.za

Appendix 2: Papanicolaou staining technique.

A 0.5% hydrochloric acid working solution is prepared by adding 5ml if 32% HCL to 995ml's of distilled water. A working solution of 1% lithium carbonate is prepared by adding 10ml of $LiCO_3$ stock solution supernatant to 990ml's of distilled water.

Staining method (staining times may vary daily due to the quality of the staining)

95% ethanol	10 minutes
80% ethanol	10 dips
50% ethanol	10 dips
Water rinse	10 dips
Hematoxylin	2-3 minutes
Water rinse	10 dips
HCl solution	2 dips
Water rinse	10 dips
Lithium carbonate solution	3 minutes
Water rinse	10 dips
95% ethanol	10 dips
Orange II	1 minute
95% ethanol	10 dips
95% ethanol	10 dips
EA 50	3 minutes
95% ethanol	10 dips
95% ethanol	10 dips
95% ethanol	10 dips
100% ethanol	10 dips
100% ethanol	10 dips
100% ethanol	10 dips
Xylene	10 dips
Xylene	10 dips
Xylene	10 dips
Coverslip	

1.2 RAPID PAPANICOLAOU STAINING METHOD (MANUAL)

Smears are fixed and dry.
Prepare staining reagents according to relevant protocol.
One dip is equivalent to 1 second in each solution.
Staining method (staining times may vary due to quality of the staining)

95% ethanol	1 minute
70 % ethanol	3-5 dips
Water	3-5 dips
Haematoxylin	45 seconds
Water	3-5 dips
95% ethanol	3-5 dips
Orange II	10 seconds
95% ethanol	3-5 dips
95% ethanol	3-5 dips
EA 50	90 seconds
95% ethanol	3-5 dips
95% ethanol	3-5 dips
100% ethanol	3-5 dips
100% ethanol	3-5 dips
Xylene	3-5 dips
Xylene	3-5 dips
Coverslip	

Appendix 3: Rapi-diff staining method

2.2 RAPIDIFF STAINING METHOD (MANUAL) – batch staining

Smears are unfixed and dry.
Staining method (staining times may vary due to quality of the staining)
One dip is equivalent to 1 second in each solution.

Methanol	5 minutes
Rdiff I (orange-red color)	20 seconds
Rdiff II (blue-purple color)	45 seconds
Water	10 dips
Water	10 dips
Allow smears to dry	
Xylene	dip
Coverslip	

2.3 EXPECTED RESULTS

Nuclei:	*Purple*
Degenerate cells:	Pink
Cytoplasm:	Pink / Blue
Red blood cells:	Pink / Red
Eosinophils:	Pink / Red

2.4 QUALITY CONTROL PROCEDURES

After a batch is stained, at least one slide must be checked for staining quality under the microscope and recorded on (APPENDIX 7).
The stain is checked uncovered while the cellular material is still wet.

- if cells appear *too dark*, slides can be differentiated in water to improve the staining quality, or if still too dark, differentiated in alcohol
- if cells appear *too light*, slides can be re-stained in the RDIFF I and RDIFF II solutions until staining quality has improved

If the QC fails, the problem is investigated and corrective action implemented accordingly:

- Re-stain slides
- Adjust staining times
- Change stains and/or solutions
- Filter stains
- Reiterate staining method to staff

3. KINYOUN STAINING METHOD (MANUAL)

The Kinyoun stain is a variation of the Ziehl-Neelsen technique. It is a method for staining acid-fast microorganisms, specifically mycobacterium.

- Soak the slides in acetone to remove the film.
- Rinse slides in water.
- Allow slides to air-dry.
- Stain the slides together with a positive quality control slide for each batch.
- Cover the slides with strips of blotting paper.
- Thoroughly moisten blotting paper with Kinyoun carbol fuchsin stain.
- Allow to stand for 5 minutes. Add more stain if the paper dries.
- Remove the blotting paper with a forceps.
- Rinse the slides with running water and drain excess water.
- Decolorize with 3% acid alcohol solution until no more stain appears in the washing (about 2 minutes).
- Wash with water.
- Counterstain with Methylene Blue (1-2 minutes).

Appendix 4: Oil red O staining technique

PURPOSE:
Demonstration of Fat, myelin lipids

RESPONSIBITY:
Special stains bench

PRINCIPLE:
The method use fat soluble dye which is more soluble in tissue fats than in dye solvents.

FIXATIVE
Fresh frozen sections or formalin fixed frozen sections. Processed tissues can't be used as the fat is dissolved out in the processing alcohols and xylene. However, there are exceptions to this in certain diseases.

PROCEDURE

1. Rinse sections in 60% isopropanol.
2. Stain in Oil Red O -- 10 min
3. Rinse off excess stain in 60% isopropanol.
4. Wash in water
5. Counter-stain with haematoxylin -- 3 min
6. Wash, blue and mount from water in P.V.P.(Polyvinyl pyrrolidine) or glycerine jelly.

RESULTS

Neutral fat droplets -- bright orange/red
Myelin lipids -- pink
Nuclei -- blue

EVALUATION
0,1 Red O consists of two components, a red oxazine which dissolves in neutral lipids and a blue oxazine which reacts with phospholipids and free fatty acids.

HAZARDS
None known

SOLUTIONS

STOCK OIL RED O
0,5g Oil Red O in 100ml concentrated isopropanol.

WORKING SOLUTION

Dilute 60ml stock with 40ml distilled water and leave to stand for 10 minutes before filtering. Stain in a closed coplin jar container, **never on a slide rack.**

REFERENCE:

Theory and practice of histological techniques by John D. Bancroft and Alan Stevens second edition

Appendix 5: Perl's Prussian blue staining technique.

A working ferrocyanide solution is prepared by adding 20ml's of 1% aqueous potassium ferrocyanide to 20ml's of 2% aqueous hydrochloric acid

PERLS PRUSSIAN BLUE - HAEMOSIDERIN / FERRIC IRON:

Principle:
Haemosiderin is a breakdown product of haemoglobin and is a protein compound. The iron-containing pigments are soluble in acids after formalin fixation. Treatment with an acid ferrocyanide solution will result in the unmasking of ferric iron in the form of the hydroxide, $Fe(OH)_3$ by dilute hydrochloric acid. The ferric iron then reacts with a dilute potassium ferrocyanide solution to produce an insoluble blue compound, ferric ferrocyanide. Distilled water and clean glassware should be used to avoid contamination.

PERLS PRUSSIAN BLUE

Method:

1. Sections to distilled water	
2. Equal amounts of 20% potassium ferrocyanide and 20% hydrochloric acid	30mins.
3. Rinse in distilled water	
4. Counterstain with 1% neutral red	2 mins.
5. Dehydrate, clear and mount	

Results:

Nuclei	-	red
Haemosiderin	-	blue/green

Precautions:

Hydrochloric acid

Causes burns
Irritating to the respiratory system
In case of contact with eyes rinse well immediately with water and seek medical advice
In case of accident or if you feel unwell, seek medical advice immediately
***Always add acid to water, never water to acid**

Ethanol / Absolute alcohol
Highly flammable
Keep container tightly closed

Xylene / Xylol
Flammable
Harmful by inhalation and in contact with skin
Avoid contact with eyes
Use in a fumehood

Appendix 6: Periodic acid-Schiff staining technique

PERIODIC ACID SCHIFFS (PAS) - GLYCOGEN:

Principle:
Periodic acid oxidizes certain tissue elements to form dialdehydes. These aldehydes react with the fuchsin-sulfurous acid which combines with the basic rosaniline to form a magenta-coloured compound.
Periodic acid is the oxidant of choice for the reaction as it does not progressively overoxidise the formed aldehydes to carboxylic acid, which would result in a weak Schiff reaction. The intensity of the reaction depends to some extent on the length of treatment with the periodic acid and Schiffs solutions.

PERIODIC ACID SHIFF'S (PAS)

Schiffs reagent:
1. Boil 200 ml distilled water and 1 g Pararosaniline (Parafuchsin hydrochloride) together
2. Cool to 50°C and filter
3. Add 20 ml 1N hydrochloric acid (98,5 ml HCl per litre)
4. Cool to 20°C
5. Add 1g anhydrous sodium metabisulphite and store in the dark overnight or until straw-coloured
6. Add 0,2g charcoal, shake vigorously and filter. Should be a colourless liquid
6. Store in the fridge

Method:
1. Take sections to distilled water
2. If a Pas with diastase digestion has been requested spit on the slide and control slide for 30 mins, if not continue from stage 4
3. Wash well after above treatment
4. 2% periodic acid — 5 mins
5. Rinse in distilled water
6. Schiff's reagent — 20 mins
7. Wash in running water — 10 mins
8. Counterstain nuclei in Haematoxylin — 2 mins
9. Blue in running tap water
10. Dehydrate, clear and mount

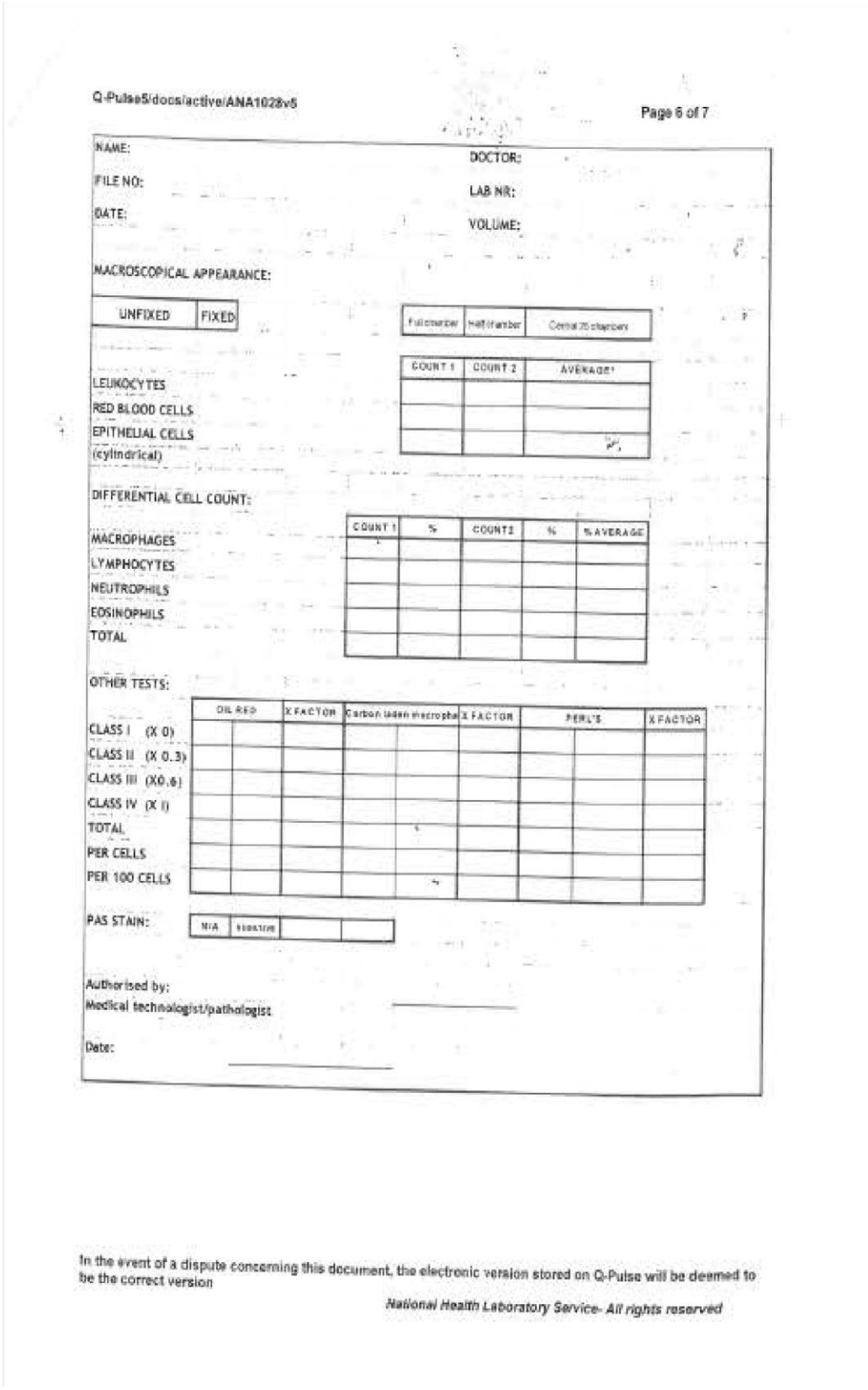
Results:
Polysacharides, mucopolysacharrides - red
Nuclei - blue

PAS DIASTASE

The presence of glycogen in a section can be shown by loss of staining after enzyme treatment when compared to the untreated test section. Positive glycogen control sections must be run with both the treated and untreated sections. Glycogen is readily digested with amylase. The most readily available amylase is saliva which contains ptyalin (salivary amylases).

Method for glycogen digestion:
1. Sections to water as usual.
2. Leave the section to remain untreated in distilled water
3. Make up a 1% diastase solution (0,5g in 50 ml dist water)
4. Place slides in a 37C incubator — 30 min
5. Wash in running water
6. Continue with the normal PAS stain from stage 4

Appendix 7: Cytology BALF differential cell and special stain quantitation form.



Q-Pulse5/docs/active/ANA1028v5

NAME:

DOCTOR:

FILE NO:

LAB NR:

DATE:

VOLUME:

MACROSCOPICAL APPEARANCE:

UNFIXED	FIXED

Full chamber	Half chamber	Central 25 chambers

	COUNT 1	COUNT 2	AVERAGE
LEUKOCYTES			
RED BLOOD CELLS			
EPITHELIAL CELLS (cylindrical)			

DIFFERENTIAL CELL COUNT:

	COUNT 1	%	COUNT 2	%	% AVERAGE
MACROPHAGES					
LYMPHOCYTES					
NEUTROPHILS					
EOSINOPHILS					
TOTAL					

OTHER TESTS:

	OIL RED		X FACTOR	Carbon laden macropha	X FACTOR	PERL'S		X FACTOR
CLASS I (X 0)								
CLASS II (X 0.3)								
CLASS III (X0.6)								
CLASS IV (X 1)								
TOTAL								
PER CELLS								
PER 100 CELLS								

PAS STAIN:	MIA			

Authorised by:
Medical technologist/pathologist

Date:

In the event of a dispute concerning this document, the electronic version stored on Q-Pulse will be deemed to be the correct version

Appendix 8(a): GeneXpert Ultra package insert.

Xpert® MTB/RIF Ultra

For *In Vitro* Diagnostic Use

Proprietary Name

Xpert® MTB/RIF Ultra

Common or Usual Name

Xpert MTB/RIF Ultra Assay

A. Intended Use

The Xpert MTB/RIF Ultra Assay, performed on the GeneXpert Instrument Systems, is a semi-quantitative, nested real-time polymerase chain reaction (PCR) *in vitro* diagnostic test for the detection of *Mycobacterium tuberculosis* (MTB) complex DNA in unprocessed sputum samples or concentrated sediments prepared from induced or expectorated sputum. In specimens where *Mycobacterium tuberculosis* complex is detected, the Xpert MTB/RIF Ultra Assay can also detect rifampin-resistance associated mutations of the *rpoB* gene.

The Xpert MTB/RIF Ultra Assay is intended for use with specimens from patients for whom there is clinical suspicion of tuberculosis (TB) and who have received no antituberculosis therapy, or less than 3 days of therapy in the last 6 months. This test is intended as an aid in the diagnosis of pulmonary tuberculosis when used in conjunction with clinical and other laboratory findings.

B. Summary and Explanation

Globally, about 2 billion people are infected with MTB.[1] In 2016, 10.4 million people developed active disease, and 1.7 million people lost their lives to the illness.[2] The route of transmission of pulmonary TB is through the air, which makes this a highly transmissible disease. Given the infectious nature of pulmonary TB, fast and accurate diagnosis is an important element of TB treatment and control.

Treatment involves prolonged administration of multiple drugs and is usually highly effective. However, *M. tuberculosis* strains may become resistant to one or more of the drugs, making cure much more difficult to achieve. Four common first-line drugs used in anti-tuberculosis therapy are isoniazid (INH), rifampin (also known as rifampicin, RIF), ethambutol (EMB), and pyrazinamide (PZA). As documented by World Health Organization, RIF resistance is rarely encountered by itself, and usually indicates resistance to a number of other anti-TB drugs.[3] It is most commonly seen in multi-drug resistant (MDR-TB) strains (defined as resistant to both RIF and INH) and has a reported frequency of greater than 95% in such isolates.[4,5,6] Resistance to RIF or other first-line drugs usually indicates the need for full susceptibility testing, including testing against second-line agents.

Molecular detection of TB and *rpoB* gene mutations associated with RIF resistance greatly reduces the time to diagnosis of both drug-susceptible and MDR tuberculosis. With the Xpert MTB/RIF Ultra Assay, this can be accomplished in unprocessed sputum samples and in prepared sediments in less than 80 minutes. The rapid detection of MTB and RIF resistance allows the physician to make critical patient management decisions regarding therapy during a single medical encounter.

Appendix 8(b): GeneXpert Ultra package insert.

C. Principle of the Procedure

The GeneXpert Instrument Systems integrate and automate sample processing, nucleic acid amplification, and detection of the target sequences in simple or complex samples using real-time PCR and melt peak detection. The system consists of an instrument, personal computer, barcode scanner, and preloaded software for running tests on patient samples and viewing the results. The system requires the use of single-use disposable GeneXpert cartridges that hold the PCR reagents and host the PCR process. Because the cartridges are self-contained, cross-contamination between samples is minimized. For a full description of the system, see the *GeneXpert Dx System Operator Manual* or the *GeneXpert Infinity System Operator Manual*.

Xpert MTB/RIF Ultra Assay includes reagents for the detection of MTB and RIF resistance and a sample processing control (SPC) to control for adequate processing of the target bacteria and to monitor for the presence of inhibitor(s) in the PCR reaction and subsequent melt peak detection. The Probe Check Control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability.

The primers in the Xpert MTB/RIF Ultra Assay amplify a portion of the *rpoB* gene containing the 81 base pair "core" region and portions of the multi-copy *IS1081* and *IS6110* insertion elements target sequences. The melt analysis with four *rpoB* probes is able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with RIF resistance. The two insertion element probes enhance the detection of *Mycobacterium tuberculosis* complex due to the multi-copy insertion element target sequences in most TB strains.

D. Reagents and Instruments

D.1 Materials Provided

The Xpert MTB/RIF Ultra Assay kit contains sufficient reagents to process 50 samples. The kit contains the following:

Xpert MTB/RIF Ultra Assay Cartridges with Integrated Reaction Tubes	**50 per kit**
• Bead 1 and Bead 2 (freeze-dried)	2 of each per cartridge
• Bead 3 (freeze-dried)	1 of each per cartridge
• Reagent 1	4 mL per cartridge
• Reagent 2	4 mL per cartridge
Sample Reagent Bottles	**50**
• Sample Reagent	8 mL per bottle
Disposable Transfer Pipettes	**60 per kit**
CD	**1**
• Assay Definition Files (ADF)	
• Instructions to import ADF into software	
• Instructions for Use (Package Insert)	

Note Sample Reagent (SR) can be colorless to yellow to amber. Color may intensify with time, but color has no effect on performance.

Note Safety Data Sheets (SDS) are available at www.cepheid.com or www.cepheidinternational.com under the **SUPPORT** tab.

Note The bovine serum albumin (BSA) in the beads within this product was produced and manufactured exclusively from bovine plasma sourced in the United States. No ruminant protein or other animal protein was fed to the animals; the animals passed ante- and post-mortem testing. During processing, there was no mixing of the material with other animal materials.

Note The transfer pipettes have a single mark representing the minimum volume of treated sample necessary to transfer to the cartridge. Use only for this purpose. All other pipettes must be provided by the laboratory.

Appendix 9: MGIT 960 package insert.

Method: as per MIC1222 (Q-Pulse/docs/active/MIC12225v9)

1. Sample reagent buffer supplied in the kit is transferred to the specimen in a 2:1 volume ratio.
2. The lid of the container is securely closed and the specimen is vortexed for 10 seconds
3. The specimen is incubated for 10 minutes at room temperature and then vortexed once again for another 10 seconds.
4. The sample is incubated at room temperature for an additional 5 minutes
5. A Xpert/MTB/RIF Ultra cartridge is labelled with the sample ID

2ml of the processed sample transferred to the Xpert/MTB/RIF Ultra cartridge and loaded into the GeneXpert instrument.

Appendix 10(a): HAIN PCR package insert.

GenoType MTBDR*plus* VER 2.0
Molecular Genetic Assay for Extraction of Bacterial DNA and Identification of the *M. tuberculosis* Complex and its Resistance to Rifampicin and Isoniazid from Clinical Specimens and Cultivated Samples

Please read the instructions on hand completely and carefully before using the kit. Strictly adhere to the established procedure to obtain correct test results.

Intended Use

The **GenoType MTBDR*plus*** VER 2.0 is a qualitative in vitro test for the extraction of bacterial DNA and the identification of the *Mycobacterium tuberculosis* complex and its resistance to rifampicin (RMP) and/or isoniazid (INH) from pulmonary smear-positive or -negative clinical specimens and cultivated samples. The following species are included in the tuberculosis (TB)-causing *M. tuberculosis* complex: *M. tuberculosis*, *M. africanum*, *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, *M. bovis* BCG, *M. microti*, *M. canettii*, and *M. pinnipedii*. The identification of RMP resistance is enabled by the detection of the most significant associated mutations of the *rpoB* gene (coding for the β-subunit of the RNA polymerase). For detection of INH resistance, the *katG* gene (coding for the catalase peroxidase) and the promoter region of the *inhA* gene (coding for the NADH enoyl ACP reductase) are examined.
The test is indicated as an aid for diagnosis and intended for use in medical laboratories.

Summary and Explanation

Tuberculosis (TB) is a bacterial infectious disease passed on by droplet infection. In 2013, there were an estimated 9.0 million incident cases of TB globally, and an estimated 1.5 million deaths occurred [1]. TB treatment requires a therapy over several months. Emergence and spread of multidrug-resistant tuberculosis (MDR-TB) is a major medical and public problem threatening global health. MDR-TB is defined as TB that is resistant at least to RMP and INH, the two most important first-line anti-TB drugs [2]. MDR-TB is a challenge to TB control due to its complex diagnosis and obstacles in treatment. In 2013, there were an estimated 480,000 cases of MDR-TB among the world's 11 million prevalent cases of TB [1].
As long as MDR-TB is not verified, use of inadequate and hence ineffective antibiotics may lead to further spread of resistant bacteria and amplification of resistance. Therefore, rapid diagnosis and identification of MDR-TB is a prerequisite for appropriate treatment.

Principles of the Procedure

The **GenoType MTBDR*plus*** test is based on the **DNA**•STRIP technology. The whole procedure is divided into three steps: (i) DNA extraction from clinical specimens (pulmonary, decontaminated) or cultured material (solid/liquid medium) – the necessary reagents A-LYS and A-NB are included in the kit, (ii) a multiplex amplification with biotinylated primers, and (iii) a reverse hybridization.
All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single-stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. Thus the probes reliably discriminate several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. A template ensures the easy and fast interpretation of the banding pattern obtained.

Storage and Disposal of Kit Constituents

1/2 Kit Component 1 of 2

2/2 Kit Component 2 of 2

Store all constituents from Kit Component 1 at 2-8°C. Store all constituents from Kit Component 2 at -20°C and keep strictly separated from contaminating DNA. Avoid repeated freezing and thawing of AM-A and AM-B; when processing only small sample numbers per run, aliquot AM-A and AM-B. Do not use the reagents beyond their expiry date. Dispose of unused reagents and waste in accordance with federal, state, and local regulations.

Precautions for Handling Kit Constituents

Observe all federal, state, and local safety and environmental regulations. Always wear suitable protective clothing and gloves.
When handling kit reagents, the following special safety measures must be applied:

Hybridization Buffer **(HYB)** and Substrate Concentrate **(SUB-C)** are not classified as hazardous. Due to their ingredients, however, hazard statement EUH210 applies: Safety data sheet available on request.

Denaturation Solution **(DEN)** contains <2% sodium hydroxide.
Warning!
H315: Causes skin irritation. H319: Causes serious eye irritation.
P280: Wear protective gloves/protective clothing/eye protection. P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P313: Get medical advice/attention.

For additional information, please refer to the safety data sheets which can be downloaded from: www.hain-lifescience.com/products/msds.html

Quality Control

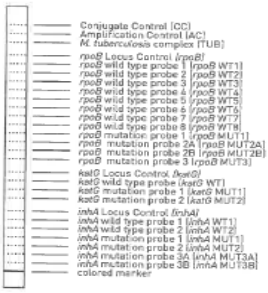
In order to control the correct performance of the test and the proper functioning of kit constituents, each strip includes 5 control zones:
- a Conjugate Control zone (CC) to check the binding of the conjugate on the strip and a correct chromogenic reaction
- an Amplification Control zone (AC) to check for a successful amplification reaction
- three Locus Control zones (*rpoB*, *katG*, and *inhA*) checking the optimal sensitivity of the reaction for each of the tested gene loci

2

Appendix 10(b): HAIN PCR package insert.

Evaluation and Interpretation of Results

Paste strips and store protected from light. An evaluation sheet is included in the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and AC with the respective lines on the sheet. For technical reasons the distances between single probes on the strips may vary slightly. **For an accurate evaluation therefore please use the provided template and align it – separately for each locus – with the respective Locus Control band.** Determine the resistance status and note down in the respective column. As a help for interpretation, evaluation examples are given in the subsequent chapter. Each strip has a total of 27 reaction zones (see figure).



Note: The strip is not displayed in original size.

Conjugate Control (CC)
A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.

Amplification Control (AC)
When the test is performed correctly, a control amplicon will bind to the Amplification Control zone.
In case of a positive test result, the signal of the Amplification Control zone can be weak or even vanish totally. This might be due to competition of the single reactions during amplification. In this case, the test was performed correctly and does not have to be repeated.
When only the CC and AC bands are developed, this represents a valid negative result. A missing AC band in case of a negative test result indicates mistakes during setup and/or performance of the amplification reaction, or presence of amplification inhibitors. In this case, the test result is not valid and the test has to be repeated with the respective sample.

***M. tuberculosis* complex (TUB)**
This zone hybridizes, as far as is known, with amplicons generated from all members of the *Mycobacterium tuberculosis* complex. If the TUB zone is negative while no evaluable resistance pattern is developed, the tested specimen does not contain bacteria belong to the *M. tuberculosis* complex and cannot be evaluated by this test system. In rare cases, the TUB zone may be negative while an evaluable resistance pattern is developed. If so, the presence of a strain belonging to the *M. tuberculosis* complex must be suspected and the test should be repeated (see below, "special case" no. 3).

Locus Controls (*rpoB*, *katG*, and *inhA*)
The Locus Control zones detect a gene region specific for the respective locus. In case of a positive test result (evaluable wild type and mutation banding pattern), the signals of the Locus Control bands may be weak.

Wild type probes
The wild type probes comprise the most important resistance regions of the respective genes (see figure 1, as well as tables 1, 2, and 3). When all wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. This indicates that the strain tested is sensitive to the respective antibiotic. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes indicates a resistance of the tested strain to the respective antibiotic.
Each pattern deviating from the wild type pattern indicates a resistance of the tested strain. The banding pattern obtained with the *rpoB* probes allows drawing a conclusion about an RMP resistance of the strain tested, the *katG* and the *inhA* banding pattern about an INH resistance.

Mutation probes
The mutation probes detect some of the most common resistance-mediating mutations (see tables 1, 2, and 3). Compared to the other probes, positive signals of the mutation probes *rpoB* MUT2A and MUT2B may show a lower signal strength.
In rare cases, when the *rpoB* MUT3 band is positive, weak staining may be detected at the *rpoB* WT8 band which is to be considered negative.
Each pattern deviating from the wild type pattern indicates a resistance of the tested strain. The banding pattern obtained with the *rpoB* probes allows drawing a conclusion about an RMP resistance of the strain tested, the *katG* and the *inhA* banding pattern about an INH resistance.

Please note:
Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone (AC) are to be considered.
Not all bands of a strip have to show the same signal strength.

Note the following special cases:
1. There is a possibility that the specimen tested contains a heteroresistant strain. In case of a heteroresistance, a mutated as well as a wild-type sequence can be detected in the respective strain; hence, one of the mutation probes as well as the corresponding wild type probe may stain positive on the respective strip. Whether the respective resistance becomes phenotypically evident depends on the ratio of mutated and nonmutated sequences at investigation.

Appendix 10(c): HAIN PCR package insert.

Determine the number of samples (number of samples to be analyzed plus control samples). Prepare the number of tubes needed. Prepare a master mix containing AM-A and AM-B and mix carefully but thoroughly (do not vortex). Alternatively, the content of an AM-A reaction tube may completely be transferred to an AM-B reaction tube. This will lead to 0.68 ml master mix for 12 amplification reactions (12 tests kit) or, respectively, 4x 1.35 ml for 4x 24 amplification reactions (96 tests kit). Please note that the master mix needs to be prepared freshly each time. Aliquot 45 µl into each of the prepared PCR tubes and add 5 µl water (molecular biology grade) to one aliquot (negative control). In a separate working area, add 5 µl DNA solution to each aliquot (except for negative control).

Amplification profile:
When using a thermal cycler from Hain Lifescience with the respective preinstallation, select protocol "MDR CUL" for cultivated samples or protocol "MDR DIR" for clinical specimens.

		Clinical specimens	Cultivated samples
15 min	95°C	1 cycle	1 cycle
30 sec 2 min	95°C 65°C	20 cycles	10 cycles
25 sec 40 sec 40 sec	95°C 50°C 70°C	30 cycles	20 cycles
8 min	70°C	1 cycle	1 cycle
Heating rate		≤ 2.2°C/sec	≤ 2.2°C/sec

Amplification products can be stored at +8 to –20°C.

Hybridization

When using a hybridization instrument from Hain Lifescience, please refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the hybridization protocol to be used.
The following protocol describes the manual hybridization using a water bath or a **TwinCubator**.

Preparation
Prewarm shaking water bath to **45°C** (the maximum tolerated deviation from the target temperature is +/–1°C) or switch on **TwinCubator**. Prewarm solutions HYB and STR to 37-45°C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary. Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer **(CON-C with CON-D, SUB-C with SUB-D)** in the amounts needed. Mix well and bring to room temperature. For each strip, add 10 µl concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

1. **Dispense 20 µl of Denaturation Solution (DEN, blue) in a corner of each of the wells used.**
2. **Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate at room temperature for 5 minutes.**
   Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. Always wear gloves when handling strips.
3. **Carefully add to each well 1 ml of prewarmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous color.**
   Take care not to spill solution into the neighboring wells.
4. **Place a strip in each well.**
   The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers, turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps.
5. **Place tray in shaking water bath/TwinCubator and incubate for 30 minutes at 45°C.**
   Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least 1/3 of its height.
6. **Completely aspirate Hybridization Buffer.**
   For example, use a Pasteur pipette connected to a vacuum pump.
7. **Add 1 ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45°C in shaking water bath/TwinCubator.**
8. **Work at room temperature from this step forward.**
   Completely remove Stringent Wash Solution.
   Pour out Wash Solution in a waste container and remove all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper. This also applies to all other wash steps.
9. **Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator (pour out RIN after incubation).**
10. **Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on shaking platform/TwinCubator.**
11. **Remove solution and wash each strip twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water (e.g. use wash bottle) on shaking platform/TwinCubator (pour out solution each time).**
    Make sure to remove any trace of water after the last wash.
12. **Add 1 ml of diluted substrate (see above) to each strip and incubate protected from light without shaking.**
    Depending on the test conditions (e.g. room temperature), the substrate incubation time, i.e. the time until the bands are clearly visible, can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.
13. **Stop reaction as soon as bands are clearly visible by briefly rinsing twice with distilled water.**
14. **Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.**

Appendix 11: Auramine O staining technique and classification

AURAMINE O METHOD

This method is used for all concentrated patient specimens. Not suitable for slides made from positive cultures.

A. REAGENTS

- **Auramine O Stain**
- **0.5% acid alcohol (Decolourizing solution)**
- **Potassium Permanganate (Counter stain)**

B. STAINING PROCEDURE

1. Fix slide with methanol for 30 seconds.
2. Flood smear with Auramine O stain (stain 1) and gently heat until steam rises. Do not overheat or boil. Stain for ±20 minutes.
3. Rinse with tap water and drain
4. Decolourise with 0.5% acid alcohol for ±2 minutes (stain 2)
5. Rinse with tap water and drain
6. Flood smear with potassium permanganate counter stain (stain 3) for ±2 minutes
7. Rinse with tap water and drain
8. Air dry - **do not blot**
9. Read as soon as possible after staining.
10. After reading, store slides immediately in slide cabinet to ensure they are not exposed to light. This is done to avoid light exposure as fluorescence fades in bright light and slides might need to be rechecked later.

C. RESULTS

Acid fast organisms emit a bright yellow fluorescence, with the potassium permanganate counterstain.
The non-specific fluorescing debris is usually a pale yellow, quite distinct from the bright yellow acid-fast organisms.

Reporting is as per the WHO/IUATLD/Stop TB, Global Laboratory Initiative partnership standardized guidelines:

Use fluorescent microscope and read slide on 40x objective.

No. of acid-fast bacilli (AFB)	Field	Report
No AFB	in one length	No acid-fast bacilli observed
1-2 AFB	in one length	Confirm with another smear
3-24 AFB	in one length	Scanty
1-6 AFB	Per field	1+
7-60 AFB	Per field	2+
>60 AFB	Per field	3+

Appendix 12: Gram stain technique and classification

Method as per MIC1208(Q-Pulse/docs/active/MIC1208v5)

1) A smear is made of the sediment of BALF following centrifugation.
2) The smear is allowed to air dry and is fixed with a low heat.
3) Methyl violet solution is applied for 30seconds.
4) The slide is rinsed with water to remove excess methyl violet solution.
5) Gram's iodine solution is applied for 30-60seconds
6) The slide is rinsed with water to remove excess gram's iodine.
7) The slide is flooded with acetone until no more colour appears to flow from the smear, 1-3seconds.
8) The slide is rinsed with water to remove excess acetone.
9) Gram's dilute carbol fuchsin is applied for 30secconds.
10) The slide is rinsed with water to remove excess carbol fuchsin.
11) The slide is carefully blotted and dried with gentle heat.
12) The smear is read microscopically using oil immersion and a 100x objective to determine the presence and type of organism.

Appendix 13: BALF microbiology culture method.

Method as per MIC1204(Q-Pulse/docs/active/MIC1204v6)

1) A purulent portion of the specimen is selected using a loop or sterile swab.
2) The various plates are inoculated with specimen are:
    - Blood agar for streptococcus pneumoniae
    - MacConkey agar for the isolation and differentiation of Gram-negative bacilli such as staphylococcus aureus and enterobacteria
    - Chocolate agar for the isolation of Haemophilus, Neisseria and Moraxella spp.
    - Columbia blood agar for the isolation of pneumococci.
3) Plates are anaerobically incubated at 35℃
4) Morphological characteristics of cultured colonies are read to determine what causative agent in
5) If morphological characteristics of the cultured colonies are indistinct, a second gram stain is performed to ascertain the micro-organism type.

Appendix 14: Sweat test processing method

Method as per CHE0705 (Q-Pulse/docs/active/CHE0705v5)

1) Filter paper is placed in 4 labelled acuvettes with lids securely closed.
2) Acuvettes are weighed and weight is recorded in grams.
3) Gauze swabs soaked with pilocarpine nitrate are placed on the anode.
4) Gauze swabs soaked in magnesium chloride are placed on the cathode.
5) Proposed sites for iontophoresis such as the inner arm are washed and dried.
6) The anode is placed on the cleaned site and the cathode is placed on the opposite surface of the arm.
7) The current is switched on and slowly increased to 4mA.
8) Current is maintained for 5 minutes, current is then slowly reduced to zero. The current is switched off and electrodes are removed.
9) After stimulation, the pilocarpine area is washed thoroughly with distilled water and dried.
10) Filter paper from an acuvette is placed on the dried area using forceps and securely covered with a square of polythene.
11) The patch is left for 30 minutes, removed with clean forceps and placed in the initial acuvette.
12) If the filter paper seems dry and the sweat weighs less than 0.05g, the area can be re-stimulated.
13) The sweat is extracted from the filter paper by adding a calibration standard 2 solution as per manufacturer's guidelines and pressed down firmly until filter paper is completely covered by the liquid.
14) The container is allowed to stand at room temperature for 2 hours.
15) The acuvettes are vortexed until the paper is thoroughly mashed.
16) The contents of the acuvette is placed into an Eppendorf and microfuged for 4 minutes at 13000rpm.
17) The supernatant is placed in the Synchron CX3 sweat analyser for chloride and sodium concentration analysis.

Appendix 15: CXR sweat test package insert

1 Introduction

1.1 Introduction

The M926S is a direct reading, digital chloride meter. It is designed for fast and accurate determinations of chloride levels in industrial samples.
Sample volume is selectable at 100µl or 20µl and results are displayed on a digital readout in mmol/l (millimoles per litre) Chloride.

This manual presents complete instructions for setting up and using the M926S. A short time spent familiarising yourself with these instructions will be more than repaid in satisfactory operation.

The M926S is intended for use by persons knowledgeable in safe laboratory practices. If the M926S is not used in accordance with these instructions for use, the protection provided by the equipment may be impaired.

A Service Manual is available as an optional extra, for use by appropriately qualified technical personnel. Please refer to Section 10.

1.2 Summary of the Test

The Sherwood Model 926S is used for the determination of chloride ions. It is an instrumental analogue of "Argentimetry", the traditional titrimetric methods using Silver Nitrate reagent. Like these classic methods it relies on the chemical formation of the very insoluble salt, Silver Chloride. The importance of chloride determination has been realised for well over a century, with many variations and changes being made to the techniques in order to improve the detectability and selectivity. Research into the analysis of chloride was conducted by Gay-Lussac (1832), Levol (1853), Mohr (1856) and Volhard (1874) and their findings have proven to be the basis of the methods, which are still in common use today.

Appendix 16: Cobas c Tina-quant IgA Gen.2 package insert.

The reagents used as per manufacturer's guidelines are the:

R1) TRIS buffer 20mmol/L, pH 8.0; NaCl 200mmol/L; polyethylene glycol: 3.6%, preservative.

R2) Anti-human IgA antibody (goat): dependent on titre; TRIS buffer: 20mmol/L, pH 8.0; NaCl: 150mmol/L; preservative.

Plasma and serum samples are collected in Li-heparin and K_2-EDTA collection tubes.

00035073431900501V10.0

IGA-2

cobas®

Tina-quant IgA Gen.2

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
03507343 190	Tina-quant IgA Gen.2 150 tests	System-ID 07 6786 7	Roche/Hitachi **cobas c** 311, **cobas c** 501/502
11355279 216	Calibrator f.a.s. Proteins (5 x 1 mL)	Code 656	
11355279 160	Calibrator f.a.s. Proteins (5 x 1 mL, for USA)	Code 656	
10557897 122	Precinorm Protein (3 x 1 mL)	Code 302	
10557897 160	Precinorm Protein (3 x 1 mL, for USA)	Code 302	
11333127 122	Precipath Protein (3 x 1 mL)	Code 303	
11333127 160	Precipath Protein (3 x 1 mL, for USA)	Code 303	
10171743 122	Precinorm U (20 x 5 mL)	Code 300	
10171735 122	Precinorm U (4 x 5 mL)	Code 300	
03121291 122	Precipath PUC (4 x 3 mL)	Code 241	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05947626 160	PreciControl ClinChem Multi 1 (4 x 5 mL, for USA)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
05947774 160	PreciControl ClinChem Multi 2 (4 x 5 mL, for USA)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:
IGA-2: ACN 458 (Standard application)
IGAP2: ACN 295 (Sensitive application)

For **cobas c** 502 analyzer:
IGA-2: ACN 8458 (Standard application)
IGAP2: ACN 8295 (Sensitive application)

Intended use

In vitro test for the quantitative determination of IgA in human serum and plasma on Roche/Hitachi **cobas c** systems.

Summary[1,2,3,4,5,6,7,8,9,10,11,12]

IgA accounts for 13 % of the plasma immunoglobulins and serves to protect the skin and mucosa against microorganisms. It is capable of binding toxins, and in combination with lysozyme develops anti-bacterial and antiviral activity. IgA is the predominant immunoglobulin in bodily secretions such as colostrum, saliva and sweat. Secretory IgA provides defense against local infections and is important in binding food antigens in the gut. In serum, IgA exists in monomeric, dimeric and trimeric forms, whereas in bodily secretions it exists exclusively in dimeric form with an additional chain (secretory component).

Increased polyclonal IgA levels may occur in chronic liver diseases, chronic infections, autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosis), sarcoidosis and Wiscott-Aldrich syndrome. Monoclonal IgA increases in IgA myeloma.

Decreased synthesis of IgA is observed in acquired and congenital immunodeficiency diseases such as Bruton type agammaglobulinemia. Reduced levels of IgA can be caused by protein-losing gastroenteropathies and loss through skin from burns.

Due to the slow onset of IgA synthesis, the IgA concentration in serum of infants is lower than in adults.

Use of specific antibodies for quantitation of serum proteins has become a valuable diagnostic tool. Light-scattering properties of antigen/antibody aggregates were first observed by Pope and Healey in 1938, and later confirmed by Gitlin and Edelhoch. Ritchie employed turbidimetric measurements to quantitate specific proteins. Quantitation of immunoglobulins can also be done using nephelometric techniques. Polymeric enhancement with polyethylene glycol (PEG) to improve sensitivity and increase the rate of antigen/antibody complex formation has been described by Lizana and Hellsing.

The Roche IgA assay is based on the principle of immunological agglutination.

In addition to the standard application (test IGA-2), there is a sensitive application (test IGAP2) designed for the quantitative determination of low IgA concentrations, e.g. in pediatric samples.

It is known that the so-called paraproteins secreted in monoclonal gammopathies (monoclonal immunoglobulinemia) may differ from the respective immunoglobulins of polyclonal origin by amino acid composition and size. This may impair the binding to antibody and hence impair accurate quantitation.

Test principle

Immunoturbidimetric assay

Anti-IgA antibodies react with antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically. Addition of PEG allows the reaction to progress rapidly to the end point, increases sensitivity, and reduces the risk of samples containing excess antigen producing false negative results.

Reagents - working solutions

R1 TRIS buffer: 20 mmol/L, pH 8.0; NaCl: 200 mmol/L; polyethylene glycol: 3.6 %; preservative; stabilizers

R2 Anti-human IgA antibody (goat): dependent on titer; TRIS buffer: 20 mmol/L, pH 8.0; NaCl: 150 mmol/L; preservative

R1 is in position B and R2 is in position C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

For USA: For prescription use only.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Danger

H318 Causes serious eye damage.

Prevention:

Appendix 17: Cobas c Tina-quant IgG Gen.2 package insert.

The reagents used as per manufacturer's guidelines are the:

R1) TRIS buffer 20mmol/L, pH 8.0; NaCl 200mmol/L; polyethylene glycol: 3.6%, preservative.

R2) Anti-human IgG antibody (goat): dependent on titre; TRIS buffer: 20mmol/L, pH 8.0; NaCl: 150mmol/L; preservative.

Plasma and serum samples are collected in Li-heparin and K_2-EDTA collection tubes.

03507432190c501V11.0

IGG-2

cobas®

Tina-quant IgG Gen.2

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
03507432 190	Tina-quant IgG Gen.2 150 tests	System-ID 07 6787 5	**cobas c** 311, **cobas c** 501/502
11355279 216	Calibrator f.a.s. Proteins (5 x 1 mL)	Code 656	
03121305 122	Calibrator f.a.s. PUC (5 x 1 mL)	Code 489	
10557897 122	Precinorm Protein (3 x 1 mL)	Code 302	
11333127 122	Precipath Protein (3 x 1 mL)	Code 303	
10171743 122	Precinorm U (20 x 5 mL)	Code 300	
03121313 122	Precinorm PUC (4 x 3 mL)	Code 240	
03121291 122	Precipath PUC (4 x 3 mL)	Code 241	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:

IGG-2: ACN 674 (Standard application for serum and plasma)

IGGC2: ACN 673 (Sensitive application for cerebrospinal fluid)

IGGU2: ACN 625 (Sensitive application for urine)

For **cobas c** 502 analyzer:

IGG-2: ACN 8674 (Standard application for serum and plasma)

IGGC2: ACN 8673 (Sensitive application for cerebrospinal fluid)

IGGU2: ACN 8625 (Sensitive application for urine)

Intended use

In vitro test for the quantitative determination of IgG in human serum, plasma, cerebrospinal fluid and urine on Roche/Hitachi **cobas c** systems.

Summary[1,2,3,4,5,6,7,8,9]

IgG molecules are composed of two light chains (kappa or lambda) and two gamma heavy chains. Approximately 80 % of serum immunoglobulin is IgG; its main tasks are the defense against microorganisms, direct neutralization of toxins and induction of complement fixation. IgG is the only immunoglobulin that can cross the placental barrier and provide passive immune protection for the fetus and newborn. This maternal protection gradually declines until the infant's own immunological system starts to develop (at about six months of age). Near-adult levels in serum/plasma are reached at 18 months.

Polyclonal IgG increases in serum/plasma may be present in systemic lupus erythematosis, chronic liver diseases (infectious hepatitis and Laennec's cirrhosis), infectious diseases and cystic fibrosis. Monoclonal IgG increases in IgG-myeloma.

Decreased synthesis of IgG is found in congenital and acquired immunodeficiency diseases and selective IgG subclass deficiencies, such as Bruton type agammaglobulinemia. Decreased IgG concentrations in serum and plasma are seen in protein-losing enteropathies, nephrotic syndrome and through the skin from burns. Increased IgG metabolism is found in Wiskott-Aldrich syndrome, myotonic dystrophy and with anti-immunoglobulin antibodies.

The determination of IgG in cerebrospinal fluid (CSF) is used for evaluation of infections involving the central nervous system (CNS), neoplasms or primary neurologic diseases (in particular, multiple sclerosis). Increased CSF IgG concentrations may occur because of either increased permeability of the blood-brain barrier or local/intrathecal production of IgG, or both.

Malfunction of the blood-brain barrier can be reliably quantified by means of the albumin CSF/serum ratio. An elevated albumin ratio is an indication of a disorder of the blood-brain barrier. If IgG and albumin are measured in CSF and serum simultaneously, differentiation between IgG originating from blood and IgG originating from intrathecal production is possible.

The determination of urine IgG aids, in combination with urinary albumin, to separate selective forms from unselective forms of tubular proteinuria, since IgG is markedly increased only in unselective forms of glomerular proteinuria (IgG/albumin > 0.03 mg/mg). Additionally, measurements of IgG in urine can be used in the monitoring and assessment of glomerular proreinuria.

The Roche IgG assay is based on the principle of immunological agglutination. In addition to the standard application (IGG-2), there are sensitive applications (IGGC2 and IGGU2) designed for the quantitative determination of IgG in CSF and urine.

It is known that the so-called paraproteins secreted in monoclonal gammopathies (monoclonal immunoglobulinemia) may differ from the respective immunoglobulins of polyclonal origin by amino acid composition and size. This may impair the binding to antibody and hence impair accurate quantitation.

Test principle

Immunoturbidimetric assay.

Anti-IgG antibodies react with antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically. Addition of PEG allows the reaction to progress rapidly to the end point, increases sensitivity, and reduces the risk of samples containing excess antigen producing false negative results.

Reagents - working solutions

R1	TRIS buffer: 20 mmol/L, pH 8.0; NaCl: 200 mmol/L; polyethylene glycol: 3.6 %; preservative; stabilizers
R2	Anti-human IgG antibody (goat): dependent on titer; TRIS buffer: 20 mmol/L, pH 8.0; NaCl: 150 mmol/L; preservative

R1 is in position B and R2 is in position C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Danger

H318 Causes serious eye damage.

Prevention:

Appendix 18: Cobas c Tina-quant IgM Gen.2 package insert.

The reagents used as per manufacturer's guidelines are the:

R1) TRIS buffer 20mmol/L, pH 8.0; NaCl 200mmol/L; polyethylene glycol: 3.6%, preservative.

R2) Anti-human IgM antibody (goat): dependent on titre; TRIS buffer: 20mmol/L, pH 8.0; NaCl: 150mmol/L; preservative.

Plasma and serum samples are collected in Li-heparin and K_2-EDTA collection tubes.

03035071901 90c501V11.0

IGM-2

cobas®

Tina-quant IgM Gen.2

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
03507190 190	Tina-quant IgM Gen.2 150 tests	System-ID 07 6788 3	Roche/Hitachi **cobas c** 311, **cobas c** 501/502
11355279 216	Calibrator f.a.s. Proteins (5 x 1 mL)	Code 656	
11355279 160	Calibrator f.a.s. Proteins (5 x 1 mL, for USA)	Code 656	
10557897 122	Precinorm Protein (3 x 1 mL)	Code 302	
10557897 160	Precinorm Protein (3 x 1 mL, for USA)	Code 302	
11333127 122	Precipath Protein (3 x 1 mL)	Code 303	
11333127 160	Precipath Protein (3 x 1 mL, for USA)	Code 303	
10171743 122	Precinorm U (20 x 5 mL)	Code 300	
03121291 122	Precipath PUC (4 x 3 mL)	Code 241	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05947626 160	PreciControl ClinChem Multi 1 (4 x 5 mL, for USA)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
05947774 160	PreciControl ClinChem Multi 2 (4 x 5 mL, for USA)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:

IGM-2: ACN 465 (Standard application)

IGMP2: ACN 274 (Sensitive application)

For **cobas c** 502 analyzer:

IGM-2: ACN 8465 (Standard application)

IGMP2: ACN 8274 (Sensitive application)

Intended use

In vitro test for the quantitative determination of IgM in human serum and plasma on Roche/Hitachi **cobas c** systems.

Summary[1,2,3,4,5,6,7,8,9,10,11,12]

IgM normally consists of 10 heavy μ-chains and 10 kappa or lambda type light chains which are always identical within a molecule. There is also a J-chain linking all the μ-chains together, so that simply speaking, IgM has a pentameric structure when compared to that of IgG. IgM is the largest immunoglobulin molecule (MW = 970000), but makes up only 6 % of the plasma immunoglobulins.

IgM is the first specific antibody to appear in the serum after infection. It is capable of activating complement, thus helping to kill bacteria. After the infection has subsided, IgM levels sink at a relatively rapid rate compared to IgG. This fact is used to advantage in the differential diagnosis of acute and chronic infections by comparing specific IgM and IgG titers. If IgM is prevalent the infection is acute, whereas if IgG predominates the infection is chronic (e.g. rubella, viral hepatitis). Increased polyclonal IgM levels are found in viral, bacterial, and parasitic infections, liver diseases, rheumatoid arthritis, scleroderma, cystic fibrosis and heroin addiction. Monoclonal IgM is increased in Waldenström's macroglobulinemia. Increased loss of IgM is found in protein-losing enteropathies and in burns. Decreased synthesis of IgM occurs in congenital and acquired immunodeficiency syndromes. Due to the slow onset of IgM synthesis, the IgM concentration in serum from infants is lower than in that from adults.

Use of specific antibodies for quantitation of serum proteins has become a valuable diagnostic tool. Light-scattering properties of antigen/antibody aggregates were first observed by Pope and Healey in 1938, and later confirmed by Gitlin and Edelhoch. Ritchie employed turbidimetric measurements to quantitate specific proteins. Quantitation of immunoglobulins can also be done using nephelometric techniques. Polymeric enhancement with polyethylene glycol (PEG) to improve sensitivity and increase the rate of antigen/antibody complex formation has been described by Lizana and Hellsing.

The Roche IgM assay is based on the principle of immunological agglutination.

In addition to the standard application (test IGM-2), there is a sensitive application (test IGMP2) designed for the quantitative determination of low IgM concentrations, e.g. in pediatric samples.

It is known that the so-called paraproteins secreted in monoclonal gammopathies (monoclonal immunoglobulinemia) may differ from the respective immunoglobulins of polyclonal origin by amino acid composition and size. This may impair the binding to antibody and hence impair accurate quantitation.

Test principle

Immunoturbidimetric assay.

Anti-IgM antibodies react with antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically. Addition of PEG allows the reaction to progress rapidly to the end point, increases sensitivity, and reduces the risk of samples containing excess antigen producing false negative results.

Reagents - working solutions

R1 TRIS buffer: 20 mmol/L, pH 8.0; NaCl: 200 mmol/L; polyethylene glycol: 3.6 %; preservative; stabilizers

R2 Anti-human IgM antibody (goat): dependent on titer; TRIS buffer: 20 mmol/L, pH 8.0; NaCl: 150 mmol/L; preservative

R1 is in position B and R2 is in position C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

For USA: For prescription use only.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Danger

H318 Causes serious eye damage.

Prevention:

P280 Wear eye protection/ face protection.

Appendix 19: Cobas c C-Reactive Protein Gen.3 package insert

The reagents used as per manufacturer's guidelines are the:

R1): $TRIS_{a)}$ buffer with bovine serum albumin; preservative.

R2): Latex Particles coated with anti-CRP(mouse) in glycine buffer; immunoglobulins(mouse); preservative.

Plasma and serum samples are collected in Li-heparin, K_2-EDTA or K_3-EDTA collection tubes.

00043568421906501V10.0

CRPL3

cobas®

C-Reactive Protein Gen.3

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
04956842 190	C-Reactive Protein Gen.3 (250 tests)	System-ID 07 6993 2	Roche/Hitachi **cobas c** 311, **cobas c** 501/502
11355279 216	Calibrator f.a.s. Proteins (5 x 1 mL)	Code 656	
11355279 160	Calibrator f.a.s. Proteins (5 x 1 mL, for USA)	Code 656	
10557897 122	Precinorm Protein (3 x 1 mL)	Code 302	
10557897 160	Precinorm Protein (3 x 1 mL, for USA)	Code 302	
11333127 122	Precipath Protein (3 x 1 mL)	Code 303	
11333127 160	Precipath Protein (3 x 1 mL, for USA)	Code 303	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05947626 160	PreciControl ClinChem Multi 1 (4 x 5 mL, for USA)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
05947774 160	PreciControl ClinChem Multi 2 (4 x 5 mL, for USA)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:

CRPL3: ACN 210

For **cobas c** 502 analyzer:

CRPL3: ACN 8210

Intended use

Immunoturbidimetric assay for the in vitro quantitative determination of CRP in human serum and plasma on Roche/Hitachi **cobas c** systems.

Summary[1,2,3,4,5,6,7,8]

C-reactive protein is the classic acute phase protein in inflammatory reactions. It is synthesized by the liver and consists of five identical polypeptide chains that form a five-membered ring having a molecular weight of 105000 daltons. CRP is the most sensitive of the acute phase reactants and its concentration increases rapidly during inflammatory processes. Complexed CRP activates the classical complement pathway. The CRP response frequently precedes clinical symptoms, including fever. In normal healthy individuals CRP is a trace protein with a range up to 5 mg/L. After onset of an acute phase response the serum CRP concentration rises rapidly and extensively. The increase begins within 6 to 12 hours and the peak value is reached within 24 to 48 hours. Levels above 100 mg/L are associated with severe stimuli such as major trauma and severe infection (sepsis). CRP response may be less pronounced in patients suffering from liver disease. CRP assays are used to detect systemic inflammatory processes; to assess treatment of bacterial infections with antibiotics; to detect intrauterine infections with concomitant premature amniorrhexis; to differentiate between active and inactive forms of disease with concurrent infection, e.g. in patients suffering from SLE or Colitis ulcerosa; to therapeutically monitor rheumatic disease and assess anti-inflammatory therapy; to determine the presence of post-operative complications at an early stage, such as infected wounds, thrombosis and pneumonia, and to distinguish between infection and bone marrow rejection. Postoperative monitoring of CRP levels of patients can aid in the recognition of unexpected complications (persisting high or increasing levels). Measuring changes in the concentration of CRP provides useful diagnostic information about how acute and how serious a disease is. It also allows judgements about the disease genesis. Persistence of a high serum CRP concentration is usually a grave prognostic sign which generally indicates the presence of an uncontrolled infection.

Test principle[9,10]

Particle enhanced immunoturbidimetric assay.

Human CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies. The aggregates are determined turbidimetrically.

Reagents - working solutions

R1 TRIS[a)] buffer with bovine serum albumin; preservatives

R2 Latex particles coated with anti-CRP (mouse) in glycine buffer; immunoglobulins (mouse); preservative

a) TRIS = Tris(hydroxymethyl)-aminomethane

R1 is in position B and R2 is in position C.

Precautions and warnings

For in vitro diagnostic use.

Exercise the normal precautions required for handling all laboratory reagents.

Disposal of all waste material should be in accordance with local guidelines.

Safety data sheet available for professional user on request.

For USA: Caution: Federal law restricts this device to sale by or on the order of a physician.

Reagent handling

Ready for use

Mix **cobas c** pack well before placing on the analyzer.

Carefully invert reagent container several times prior to use to ensure that the reagent components are mixed.

Storage and stability

CRPL3

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Diluent NaCl 9 %

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or sample collection containers.

Only the specimens listed below were tested and found acceptable.

Serum.

Plasma: Li-heparin, K_2-EDTA, K_3-EDTA plasma

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Appendix 20(a): Anyplex® II RV16 PCR system package insert.

Samples should be transported in viral transport medium, if not, viral transport medium is to be added to the sample. . Samples are processed at Tygerberg virology as per VIR0619(QPulse5/docs/active/VIR0619v3 standard operating procedure.)

PRINCIPLES AND PROCEDURE OVERVIEW

1. Principles

Anyplex™ II RV16 Detection (V1.1) exhibits Seegene's proprietary TOCE™ technology, which allows detection of multiple pathogens in a single fluorescence channel on real-time PCR instruments.

In current melting curve analysis, differences in melt peak temperatures (Tm) are often observed among DNAs with high sequence variations, raising issues in the field of clinical diagnostics where accurate and reproducible test results are critical. However, TOCE™ technology is not affected by sequence variations, guaranteeing consistent Tm values.

Anyplex™ II RV16 Detection (V1.1) can perform multiplex examination either by End point-CMTA (End point-Catcher Melting Temperature Analysis) or cyclic-CMTA (cyclic-Catcher Melting Temperature Analysis) method. The cyclic-CMTA method which represents a new class of multiplex molecular test.

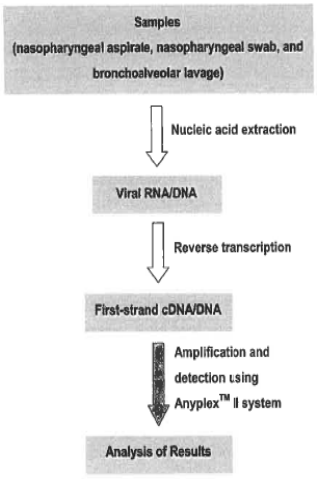
Anyplex™ II RV16 Detection (V1.1) is a high multiplex real-time PCR assay that permits simultaneous amplification, detection and differentiation of target nucleic acids of influenza A virus (Flu A), influenza B virus (Flu B), human respiratory syncytial virus A (RSV A), human respiratory syncytial virus B (RSV B), human metapneumovirus (MPV), human adenovirus (AdV), human coronavirus 229E (229E), human coronavirus NL63 (NL63), human coronavirus OC43 (OC43), human rhinovirus A/B/C (HRV A/B/C), human enterovirus (HEV), human bocavirus 1/2/3/4 (HBoV), and human parainfluenza virus 1 (PIV1) , human parainfluenza virus 2 (PIV2), human parainfluenza virus 3 (PIV3), human parainfluenza virus 4 (PIV4) and Internal Control (IC).

In PCR, amplification efficiency is often reduced by inhibitors present in clinical specimens. Therefore, Internal Control (IC) is incorporated into the product as an exogenous whole process control to monitor nucleic acid extraction and to check for possible PCR inhibition. IC is co-amplified with target nucleic acids within the clinical specimens.

To prevent amplification product acting as potential contaminants, Uracil-DNA glycosylase (UDG)-dUTP system is employed in Anyplex™ II RV16 Detection (V1.1). The UDG-dUTP system is commonly used when performing PCR to eliminate amplicon carry-over using UDG excises uracil residues from DNA by cleaving the N-glycosylic bond.

Seegene Anyplex™ II RV16 Detection (V1.1)

2. Procedure Overview



< Anyplex™ II RV16 Detection (V1.1) procedure overview >

Appendix 20(b): Anyplex® II RV16 PCR system package insert.

- Prepare and use a different pipette set for each of the following areas: nucleic acid extraction, reagent mixing, nucleic acid template addition, and handling of PCR products.
- Open PCR reaction tubes or strips after amplification only at designated spaces, to avoid contamination of the area with amplicons.
- Store positive materials separated from the kit's reagents.
- Laboratory safety procedures (refer to Biosafety in Microbiological and Biomedical Laboratories & CLSI Documents) must be taken when handling specimens. Thoroughly clean and disinfect all work surfaces with 0.5% sodium hypochlorite (in de-ionized or distilled water). Product components (product residuals, packaging) can be considered as laboratory waste. Dispose of unused reagents and waste in accordance with applicable federal, state, and local regulations.
- Expiry date is 12 months at ≤ -20℃ from the date of manufacture. Please refer to label for final expiry date.

Seegene Anyplex™ II RV16 Detection (V1.1)

INTENDED USE

Anyplex™ II RV16 Detection (V1.1) is a qualitative *in vitro* test for detection of respiratory viruses from nasopharyngeal aspirate, nasopharyngeal swab, and bronchoalveolar lavage specimens.

Anyplex™ II RV16 Detection (V1.1) consist of two PCR reactions (A set and B set).

A set is a multiplex assay that permits the simultaneous amplification of target nucleic acid of 8 respiratory viruses.

B set is a multiplex assay that permits the simultaneous amplification of target nucleic acid of 8 respiratory viruses.

A set	B set
Influenza A virus (Flu A)	Human respiratory syncytial virus A (RSV A)
Influenza B virus (Flu B)	Human respiratory syncytial virus B (RSV B)
Human adenovirus (AdV)	Human metapneumovirus (MPV)
Human parainfluenza virus 1 (PIV1)	Human coronavirus 229E (229E)
Human parainfluenza virus 2 (PIV2)	Human coronavirus NL63 (NL63)
Human parainfluenza virus 3 (PIV3)	Human coronavirus OC43 (OC43)
Human parainfluenza virus 4 (PIV4)	Human bocavirus 1/2/3/4 (HBoV)
Human rhinovirus A/B/C (HRV A/B/C)	Human enterovirus (HEV)

Flu A: The primers detect various subtypes of influenza A virus including the H5N1 subtype which causes highly pathogenic avian influenza and the novel influenza A virus (H1N1 subtype) of swine origin that was firstly detected in April, 2009.

AdV: The primers are designed to detect almost of B, C, and E types and some of A, D, and F types of human adenovirus.

HEV: The primers are designed to detect almost of A, B types and some of C, D types of human enterovirus.

Appendix 21(a): : REALSTAR CMV PCR KIT package insert

Samples should be transported in viral transport medium, if not, viral transport medium is to be added to the sample. Samples are processed at Tygerberg virology as per VIR0624 (Q-Pulse5/docs/active/VIR0624v3 standard operating procedure.)

5. Background Information

The human Cytomegalovirus (CMV) is a member of the family *Herpesviridae* and belongs to the subfamily *betaherpesvirinae*. It consists an icosahedral capsid with a linear double-stranded DNA genome of approximately 230 kbp, a surrounding integument and an outer envelope.
CMV has a worldwide distribution and infects humans of all ages, with no seasonal or epidemic patterns of transmission. The seroprevalence of CMV increases with age in all populations and ranges from 40 to 100%. Similar to infections with other herpesviruses, primary infection with CMV results in the establishment of a persistent or latent infection. Reactivation of the virus can occur in response to different stimuli, particularly immunosuppression. The vast majority of CMV infections are asymptomatic or subclinical, but congenital infections and infections in immunocompromised patients may be symptomatic and serious. In immunocompromised hosts, such as transplant recipients, HIV-infected or cancer patients, a CMV infection or reactivation may become a life-threatening disseminated disease.

6. Product Description

The RealStar® CMV PCR Kit 1.0 is an *in vitro* diagnostic test, based on real-time PCR technology, for the detection and quantification of CMV specific DNA. The assay includes a heterologous amplification system (Internal Control) to identify possible PCR inhibition and to confirm the integrity of the reagents of the kit.

The test is based on real-time PCR technology, utilizing polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified DNA. The probes are labelled with fluorescent reporter and quencher dyes.

RealStar® CMV PCR Kit 1.0

Probes specific for CMV DNA are labelled with the fluorophore FAM. The probe specific for the Internal Control (IC) is labelled with the fluorophore JOE. Using probes linked to distinguishable dyes enables the parallel detection of CMV specific DNA and the Internal Control in corresponding detector channels of the real-time PCR instrument.

The test consists of two processes in a single tube assay:

- PCR amplification of target DNA and Internal Control
- Simultaneous detection of PCR amplicons by fluorescent dye labelled probes

The RealStar® CMV PCR Kit 1.0 was developed and validated to be used with the following real-time PCR instruments:

- Mx 3005P™ QPCR System (Stratagene)
- VERSANT™ kPCR Molecular System AD (Siemens)
- ABI Prism® 7500 SDS and 7500 Fast SDS (Applied Biosystems)
- LightCycler® 480 Instrument II (Roche)
- Rotor-Gene™ 3000/6000 (Corbett Research)
- Rotor-Gene Q 5/6 plex Platform (QIAGEN)

Appendix 21(b): : REALSTAR CMV PCR KIT package insert

8. Instructions for Use

8.1 Sample Preparation

Extracted DNA is the starting material for the RealStar® CMV PCR Kit 1.0. The quality of the extracted DNA has a profound impact on the performance of the entire test system. It has to be ensured that the system used for nucleic acid extraction is compatible with real-time PCR technology.

The following nucleic acid extraction kit was validated for use with the RealStar® CMV PCR Kit 1.0:

- QIAamp® MinElute® Virus Spin Kit (QIAGEN)

In order to increase the sensitivity of the system, the protocol of the QIAamp® MinElute® Virus Spin Kit (QIAGEN) can be modified according to the specifications listed in Table 3: Adaptations of the QIAamp® MinElute® Virus Spin Kit (QIAGEN) protocol (page 23).

If using a spin column based sample preparation procedure including washing buffers containing ethanol, an additional centrifugation step for 10 min at approximately 17000 x g (~ 13000 rpm), using a new collection tube, prior to the elution of the nucleic acid is highly recommended.

NOTE

⚠ ***The use of carrier RNA is crucial for extraction efficiency and stability of the extracted nucleic acid.***

⚠ ***Ethanol is a strong inhibitor in real-time PCR. If your sample preparation system is using washing buffers containing ethanol, you need to make sure to eliminate any traces of ethanol prior to elution of the nucleic acid.***

For additional information and technical support regarding pre-treatment and sample preparation please contact our Technical Support:

e-mail: **support@altona-diagnostics.com**
phone: **+49-(0)40-5480676-0**

8.2 Master Mix Setup

All reagents and samples should be thawed completely, mixed (by pipetting or gentle vortexing) and centrifuged briefly before use.

The RealStar® CMV PCR Kit 1.0 contains a heterologous Internal Control (IC), which can either be used as a PCR inhibition control or as a control of the sample preparation procedure (nucleic acid extraction) and as a PCR inhibition control.

- If the IC is used as a PCR inhibition control, but not as a control for the sample preparation procedure, the Master Mix is set up according to the following pipetting scheme:

Number of Reactions (rxns)	1	12
Master A	5 µl	60 µl
Master B	15 µl	180 µl
Internal Control	1 µl	12 µl
Volume Master Mix	21 µl	252 µl

- If the IC is used as a control for the sample preparation procedure and as a PCR inhibition control, the IC has to be added during the nucleic acid extraction procedure.

Appendix 22: : Haemocytometer

Appendix 23: : Cytology BAL cell count dilutions

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CYTOCENTRIFUGE FOR BRONCHO-ALVEOLAR LAVAGES.

Fix the specimen with an equal volume of histological phosphate-buffered buffered formalin. If there are a lot of small cells (lymphocytes or neutrophils), you can use a slightly larger volume for smear.

UNFIXED: CELLS/ML ($X10^3$)	FIXED: CELLS/ML ($X10^3$)	DROPS	MICRO-L	PAPERS
BEFORE FIXING	AFTER FIXING			
50 -60	25-30	14	434	2
60-80	30-40	13	403	2
80-100	40-50	11	341	2
100-120	50-60	9	279	2
120-160	60-80	7	217	2
160-240	80-120	5	155	1
240-300	120-150	4	124	1
300-380	150-190	3	95	1
380-440	190-220	2	90	1

1 – 24 $X10^3$ (very low counts): Take 8 ml BAL (filtered), add 2 ml formalin.
Make 4 smears with 14 drops & 2 papers. Count 100 cells on each of 2 smears.

440 000 – 880 000 cells/ml: dilute with normal saline as follows:
2ml BAL + 2ml saline + 4 ml formalin (i.e. 4x dilution, or double the usual dilution.)

UNFIXED: CELLS/ML ($X10^3$)	FIXED: CELLS/ML ($X10^3$)	DROPS	MICRO-L	PAPERS
BEFORE FIXING	AFTER FIXING			
440-460	110-115	5	155	1
460-600	115-150	4	124	1
600-740	150-185	3	95	1
740-880	185-220	2	90	1

880 000 – 2000 000 cells/ml: dilute with normal saline as follows:
1ml BAL + 3ml saline + 4 ml formalin (i.e. 8x dilution)

UNFIXED: CELLS/ML ($X10^3$)	FIXED: CELLS/ML ($X10^3$)	DROPS	MICRO-L	PAPERS
BEFORE FIXING	AFTER FIXING			
880-920	110 -115	5	155	1
920-1200	115 -150	4	124	1
1200-1600	150 -200	3	95	1
2000 -2400	200 -300	2	90	1

PTO:

25 central = X 10
1/2 = ÷ 0,45
whole = ÷ 0,9

Appendix 24: : Cytology BAL procedure

METHOD:

TOTAL CELL COUNT:

- Use the specific BAL form to record the date, specimen number, patient name, clinician's name, volume and macroscopic appearance. Indicate whether the sample is fixed or unfixed.
- Vortex or gently invert specimen to re-suspend content.
- Aliquot 13µl or 1 drop of sample fluid with a semi-automatic pipette or standard 3ml pipette on to each of the two counting chambers of the haemocytometer. Cover with a coverslip.
- Quantify the contents of each of the 2 chambers on the designated BAL microscope using the 40x lens.
- Count leucocytes, red blood cells and cylindrical bronchial epithelial cells.
- Indicate whether the count was done on full, half chamber or using 25 central squares.
- Record the results and calculate the average of the 2 separate counts.
- Use this figure to determine what amount of saline and formaline to use for specimen fixation and dilution if necessary.
- Log the BAL in the BAL log book, indicate the date, the patient's name, accession number, who performed the test, the cell count as well as volume and number of filter cards used when preparing the cytospin slides.
- Use a specific calculation to calculate the amount of cells per ml x 10^3 found in the BAL file.
- Aliquot specimen into 2 labelled specimen jars, labelled with patient name, accession number and type of fixative.
- One jar must contain a formalin fixed specimen and the other must contain a cyto-rich red fixed specimen. The specimens is fixed with formalin to prepare ORO stain, and cyto rich red preservative fluid to fix the specimen which will be used to make the 1P, 1Rapidiff, Perl's and PAS stained slides respectively.
- After dilution and fixing, refrigerate the solution for at least 30 minutes before making smears. 22/8/2020
- Samples with total average of <50x10^3 are considered as a BAL reject, as no further requested tests can be performed on the specimen. "Reject" and the average x10^3 must be logged into the BAL log book. The code of "UN48" which reads "Too few cells present for a reliable cytological diagnosis" is to be used. The sentence "Too few cells present to perform differential count and special stains" is to be entered under the additional findings tab on Trakcare.
- The recommendations: "SA29" repeat if clinically indicated and "SA04" clinical management advised are to be used.
- The inadequate case with the initial cell counts are to be shown to be taken to a checker technologist and deemed inadequate as a secondary measure for quality control purposes. The case is to be fully authorized by the designated medical technologist.

PREPARATION:

- 8 Slides are to be prepared, 1PAP, 1MGG, 2PERLS, 2ORO and 2PAS.
- Label the slides with the patient's name, accession number and type of stain.
- Place the labeled slides in a cytospin slide holder, place filter card and chamber on top and clip in position.
- A specific amount of specimen is used according to amount of cells and dilution which is found in the BAL file located at the BAL station in the general lab.
- Cytospin at 1000rpm for 10 minutes.
- The Pap and Rapi-diff stains are performed by the general lab and the ORO stain is performed by the designated technologist in the cytology laboratory.

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