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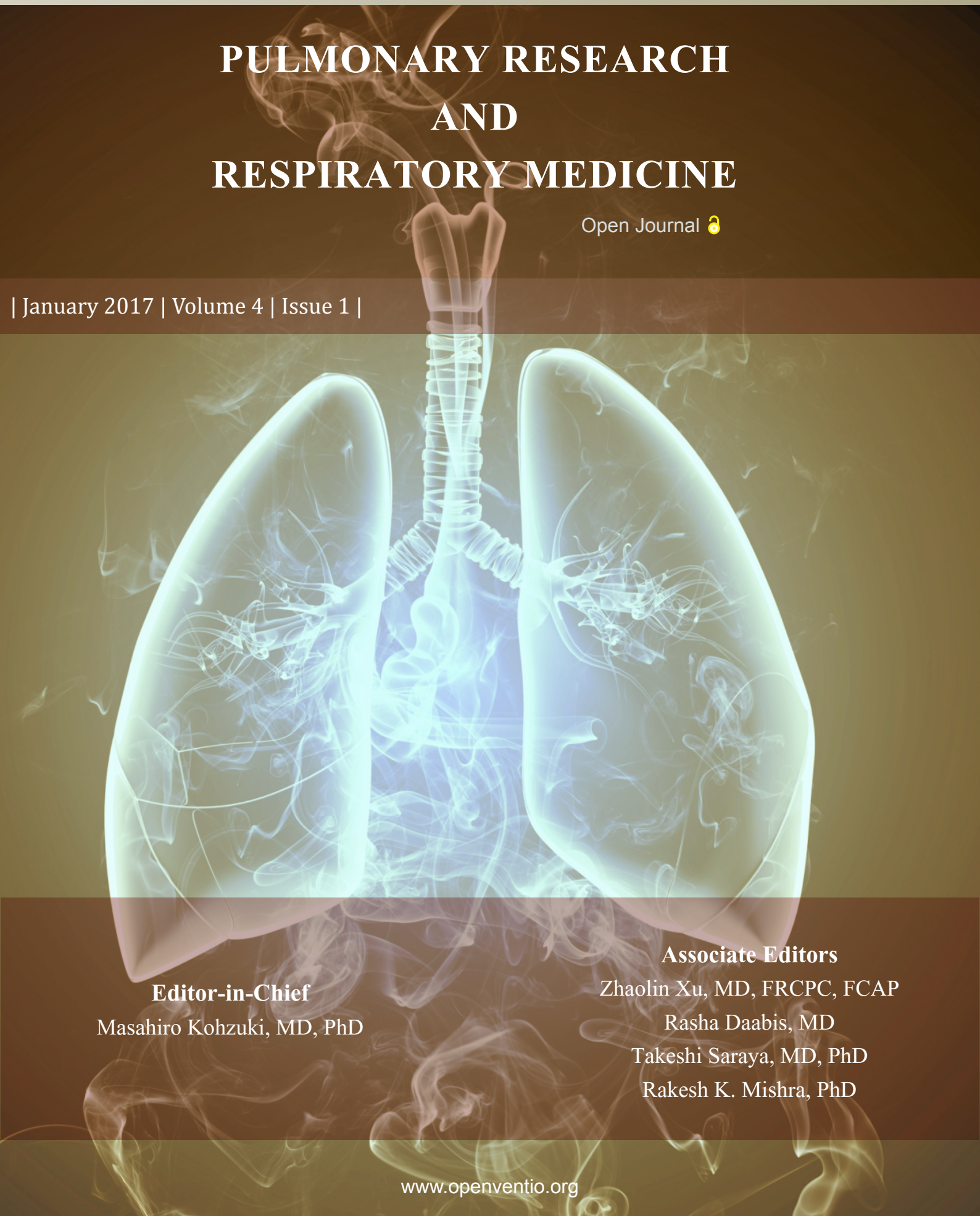

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Mini Review

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Tuberculosis in Pregnancy: Delayed Diagnosis, Lost Lives

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ABSTRACT

Tuberculosis still remains an important global health disease, killing many people annually. Yet it is a preventable cause of death. Maternal mortality rates due to tuberculosis and HIV/AIDS in Sub-Saharan Africa make up a significant proportion of maternal deaths. It remains a disease of poverty, overcrowding and underdevelopment. Pregnancy increases maternal and fatal mortality in AIDS-infected women. In pregnancy it can be a challenging diagnosis to make, delaying treatment that could lead to adverse outcomes. Clinicians working in high prevalence areas of tuberculosis and those looking after immunocompromised patients should maintain a high index of suspicion. Developing countries must be helped to develop by the world financial institutions with debt reduction. The Sustainable Development Goal aims to end tuberculosis related deaths, transmission and catastrophic costs by 2030. Tuberculosis is a preventable death, the world must act together to prevent unnecessary deaths.

KEYWORDS: Tuberculosis; Pregnancy; Complications; Maternal mortality; Outcomes.

INTRODUCTION

Every year around 250,000 women die during pregnancy and childbirth. Maternal mortality rates due to tuberculosis and HIV/AIDS in Sub-Saharan Africa now supercede obstetric deaths.[1] These infections can make up to 28% of maternal deaths.[2] Maternal tuberculosis can remain unrecognised and an underestimated tragedy.[3] Tuberculosis still remains the leading infectious cause of death in women worldwide, creating orphans, impoverished families and reduces the economic development of society. Approximately, a third of the world population are infected with tuberculosis, which still remains a major cause of preventable death in women. It kills more women each year than any other infection.[4] Untreated tuberculosis causes poor maternal and fetal/neonatal outcomes.[5]

In pregnancy it can be a challenging diagnosis to make delaying treatment that could lead to poor adverse outcomes. Clinicians working in high tuberculosis areas and those looking after immunocompromised patients should maintain a high index of suspicion.

MICROSCOPY AND PATHOLOGY

The majority of cases are caused by Mycobacterium *tuberculosis* in 95% of cases and Mycobacterium *bovis* in 5% of cases.[6] Tuberculosis is an airborne disease spread mainly by droplets during coughing, sneezing, talking or breathing in overcrowded environments. The primary focus is the lungs and from there it can spread haematogenously to become disseminated throughout the body.

Tuberculosis is generally a disease of poverty and overcrowding. The HIV/AIDS pandemic has contributed to the surging cases of tuberculosis. The combination of pregnancy, tuberculosis and HIV/AIDS exposes pregnant women to high risks of mortality. A person with

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both HIV and tuberculosis infection is thirty times more likely to become ill with tuberculosis than a person with tuberculosis infection alone.[7]

CLINICAL FEATURES

Tuberculosis in pregnancy can present with vague symptoms, some of them mimicking normal physiology[8] of pregnancy hence leading to delayed diagnosis and treatment. Patients may present with a productive or non-productive cough, chest pains, haemoptysis and generalised body weakness. They may also present with low-grade fever, headaches and visual disturbances. Other symptoms may include loss of appetite, nausea and vomiting, bone pain and swollen lymph glands depending on the main focus of infection. HIV-infected patients have depressed defence mechanisms and may present with disseminated tuberculosis with little or no clinical signs or symptoms.

DIAGNOSIS

There is usual delay in diagnosis of tuberculosis in pregnancy.[8,9] Therefore a high clinical index of suspicion must be maintained especially among vulnerable groups such as those infected with HIV/AIDS. In patients with productive coughs sputum should be sent for Acid-Alcohol-Fast Bacilli test and histological examination with Ziehl-Neelsen staining. Red Acid-Alcohol Fast Bacilli can be seen in Ziehl-Neelsen stain (Figure 1). Culturing the bacilli in egg-based medium like Lowenstein-Jensen medium takes 4-6 weeks. In HIV-infected patients, sputa are usually negative for Alcohol-Fast Bacilli causing diagnostic difficulty and delayed treatment. In some studies a significantly higher proportion of bacterially confirmed pulmonary tuberculosis patients were HIV co-infected patients than HIV negative tuberculosis patients.[10,11]

A full blood count is recommended as some patients may be anaemic due to chronic ill-health. Those not yet screened for HIV must have this test done as the two conditions usually coexist.[7] A chest X-ray with abdominal shielding must be done which may reveal pulmonary tuberculosis. Typical chest X-ray findings include military picture (Figure 2) and cavitations (Figure 3). Enlarged lymph nodes can have fine needle aspiration for histological examination. If there are signs and symptoms suggestive of tuberculuos meningitis a lumbar puncture should be done and the cerebrospinal fluid sent for microscopy and culture. Tuberculosis screening as part of antenatal care in high prevalence regions may be helpful[12] to pick up latent tuberculosis. Early diagnosis would reduce morbidity and mortality rates.

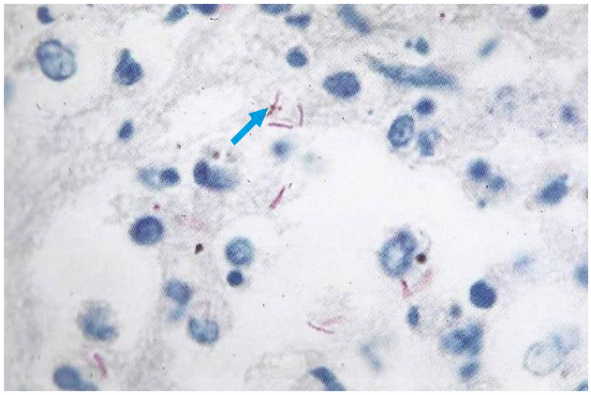

Figure 1: Red acid-alcohol fast bacilli in a Ziel-Neelsen stain showing the causative organism for tuberculosis found in a sputum sample.

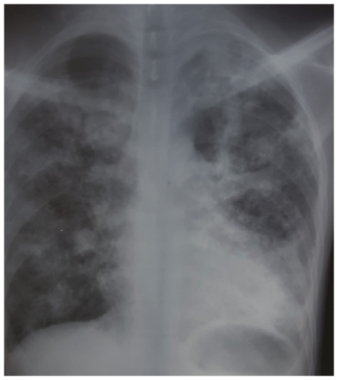

Figure 2: A chest X-ray showing military tuberculosis.

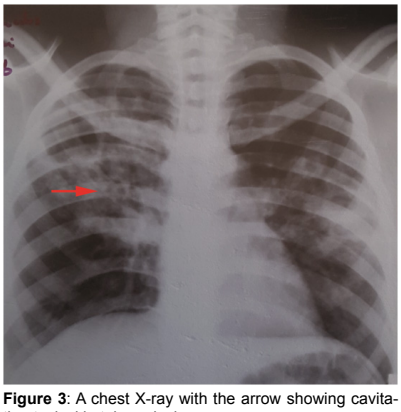

Figure 3: A chest X-ray with the arrow showing cavitation typical in tuberculosis.

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MATERNAL COMPLICATIONS

Tuberculosis is an overwhelming illness on the body, causing wasting, generalised body weakness and exposes pregnant women to further risks of other infections. Tuberculosis can be pulmonary, extrapulmonary,[13] in lymph nodes, intestines, peritoneum, bones, kidneys and meninges. Extrapulmonary tuberculosis does affect pregnancy adversely.[14] The complications of tuberculosis include chronic anaemia, threatened miscarriage, premature prelabour rupture of membranes[15] and preterm labour. Pregnant women are more likely to experience chorioamnionitis,[16] postpartum anaemia, blood transfusion, pneumonia, acute respiratory distress syndrome and mechanical ventilation. Maternal deaths may result from overwhelming and disseminated disease, pulmonary compromise or meningitis. The patients that are HIV co-infected have more complications than those that are HIV negative.[16]

There may be intrauterine growth restriction, fetal distress, chorioamnionitis[17] and intrauterine death. There can be rarely congenital tuberculosis.[18] There appears that there are more congenital abnormalities in neonates born to mothers suffering from tuberculosis.[16] Open pulmornary tuberculosis poses a grave danger to the neonate born to a mother suffering from the disease. Pregnancy increases maternal and fetal mortality in AIDS-infected women.[19]

MANAGEMENT

The effective management of tuberculosis during pregnancy is a multi-disciplinary process involving the obstetrician, paediatrician, tuberculosis specialist, public health specialist[20] and laboratory scientist. Patients with open disease should be nursed in an isolation ward until they are rendered sputum negative usually after 2 weeks of chemotherapy. Six weeks combination drug regimes are usually curative. Directly observed therapy, short-course, is a tuberculosis control measure recommended by the World Health Organisation (WHO). The drug combination of rifampicin, isoniazid and ethambutol has been widely used in pregnancy with little fatal teratogenic effects being reported.[21] Neonates born to mothers that are still sputum positive receive isoniazid therapy until their mothers are rendered sputum negative.

Contact tracing of all cases of open tuberculosis are mandatory. Tuberculosis is a notifiable public health disease right up to the World Health Organisation (WHO).

MODE OF DELIVERY

Patients suffering from tuberculosis are delivered as per other obstetric patients. The indications for Caesarean deliver are as for normal obstetric indications. If they are delivered by an abdominal route before they are rendered sputum negative, the usual precautions taken for the care of infected cases in theatre are followed. If they have a normal vaginal delivery, the usual precautions of dealing with infectious disease cases should be taken by the health care staff such as wearing face masks for their own protection.

OUTCOMES

Tuberculosis is a largely treatable disease. Fetal outcomes are good after successful treatment.[22,23] Meningeal tuberculosis is one of the major causes of maternal deaths as it is of insidious[24] onset hence early recognition and treatment improves outcomes. Breastfeeding is safe unless otherwise contraindicated.

CONCLUSIONS

Developing countries must take considerable appropriate action soon[25] to prevent escalating tuberculosis rates. The Sustainable Development Goals aim to end tuberculosis related deaths, transmission and catastrophic costs by 2030.[26] Achieving 90-90-90 targets for tuberculosis that is 90% vulnerable population screened, 90% diagnosed and started on treatment and at least 90% cured can be a goal to aim at.

COMPETING INTERESTS: None.

AUTHOR'S CONTRIBUTION

This is the sole work of Mr. S. Ngwenya.

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Mini Review

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Pneumocystis Carinii Pneumonia; Lost Lives in Pregnancy: Chemoprophylaxis Saves Lives

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ABSTRACT

The advent of HIV/AIDS brought opportunistic infections such as *Pneumocystis jiroveci pneumonia* (PJP), formerly known as *Pneumocystis carinii pneumonia* (PCP) to the fore. This is a fungal opportunistic infection that is an human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) defining illness. It poses significant risks to both the mother and the fetus. Pneumocystis carinii pneumonia can have a sub-clinical insidious onset but can rapidly progress to acute respiratory failure leading to maternal and fetal demise. The best form of management of pneumocystis carinii pneumonia is prevention. Widespread chemoprophylaxis with co-trimoxazole reduces morbidity and mortality. The management calls for a multidisciplinary approach involving the physician, obstetrician and pneumologist. Pneumocystis carinii pneumonia has a more aggressive course during pregnancy with increased morbidity and mortality, maternal and fetal outcomes remaining dismal. Therefore, it is important that widespread HIV testing and use of co-trimoxazole chemoprophylaxis are made readily available to reduce morbidity and mortality. It must be a differential diagnosis of any pregnant HIV seropositive patient presenting with pulmonary symptomatology.

KEYWORDS: Pneumocystis carinii pneumonia; Pregnancy; HIV/AIDS; Maternal mortality; Co-trimoxazole; Outcomes.

INTRODUCTION

Pneumocystis carinii pneumonia is spread by the airborne route. It caused significant morbidity and mortality in the early stages of the HIV/AIDS pandemic before the advent of anti-retroviral chemotherapeutic agents. It is now decreasing in incidence due to anti-pneumocystis[1] therapy but still causes maternal deaths in HIV infected pregnant mothers. Historically, pneumonia during pregnancy has been associated with increased morbidity and mortality compared to non-pregnant women.[2] Acute lung injury during pregnancy results in high morbidity and mortality for both the mother and the fetus.[3] In the USA, *Pneumocystis carinni pneumonia* is the most common cause of AIDS-related death in pregnant women.[4] Many HIV-infected persons in the USA remain at high risk of opportunistic infection because they are unaware of their HIV infection.[5]

It therefore remains a subject of great importance to the population especially in areas with high prevalence of HIV/AIDS. There is a proven chemoprevention agent that is safe and reduces morbidity and mortality in HIV/AIDS patients. All efforts must be made to save lives of pregnant women regardless of their HIV serostatus.

PATHOGENESIS

The causative infective agent is the unicellular *Pneumocystis jirovecii*. This is a common fungus

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found in the environment and rarely causes disease in healthy people. However in immune-compromised patients, like HIV/AIDS and cancer patients it causes pneumonia. The fungus can colonise the oropharyx, trachea, trachea-bronchial, bronchial, alveoli and lung parenchyma areas. The microorganism can spread throughout the lungs.

Most pneumocystosis pathophysiological changes result from the parasite's attachment and proliferation in the lungs, resulting in a filling of alveoli with masses of the micro-organism.[6] A host response takes place by hypertrophy and hyperplasia involving type 2 epithelial alveolar cells. The effect of this is interference in pulmonary gaseous exchange leading to respiratory compromise and failure.

CLINICAL PRESENTATION

Pneumocystis carinii pneumonia can have a sub-clinical insidious onset but can rapidly progress to acute respiratory failure[7] leading to the death of the mother[8] and the fetus. Patients may present with cough, chest pains and worsening shortness of breath.[9] Clinically, they may be tachypnoic, tachycardic, cyanosed and respiratory distress. It must be considered as a differential diagnosis in all HIV seropositive patients complaining of chest problems.

The investigations such as arterial blood gases reveal profound hypoxia in *Pneumocystis carinii pneumonia* patients. The chest-x-ray is typically described as showing a diffuse interstial pneumonia (Figure 1). The chest-x-ray may also reveal pneumothoraces or pleural effusions. Ultrasonographic appearances include consolidation with dynamic air bronchograms and pleural effusions.[10]

Definitive diagnosis is with molecular tests such as polymerase chain reaction (PCR). Obtaining specimens for such testing may be difficult.[11] The diagnosis is confirmed by the detection of trophozoides and/or cysts of *Pneumocystis jirovecii* in bronchoalveolar lavage samples by using several staining techniques.[1] The use of PCR with the standard immunofluorescent or colorimetric tests allows rapid and accurate diagnosis. This greatly improves the commencement of chemotherapeutic agents and may improve outcomes. Bronchoalveolar lavage samples can be obtained by rigid or flexible bronchoscopy. Besides broncoalveolar lavage samples other samples that can be tested include oropharyngeal wash, sputum and blood.[11]

MANAGEMENT

The best form of management of pneumocystis carinii pneumonia is prevention. Widespread chemoprophylaxis with a daily dose of co-trimoxazole (trimethoprim-sulfamethoxazole) is now recommended by the World Health Organization (WHO) for all HIV seropositive patients.[12,13] A systematic review of the literature has concluded that it is safe to give in pregnant and breastfeeding HIV seropositive patients.[14] The fetal adverse effects are outweighed by the benefits hence co-trimoxazole is given in pregnancy.

The management calls for a multidisciplinary approach involving the physician, obstetrician and pneumologist. Pregnant patients presenting in acute state present a difficult dilemma to the clinician. There could be a high possibility of immediate maternal and fetal poor outcomes. The treatment involves oxygen therapy and high doses of co-trimoxazole. The other drug that can be used is pentamidine in combination with co-trimoxazole. Pentamidine can be given in an aerosolized form.[15]

Those patients presenting with pneumothoraces with respiratory distress will need urgent tube thoracostomy to relieve the pneumothoraces and respiratory distress. Patients may need to be admitted to the intensive care unit (ICU) for ventilatory support. Difficult decisions about delivery may have to be taken, at times compromising fetal health if the fetus is premature. Delivery may improve ventilation and improve maternal outcomes. Those collapsing patients with live fetuses should

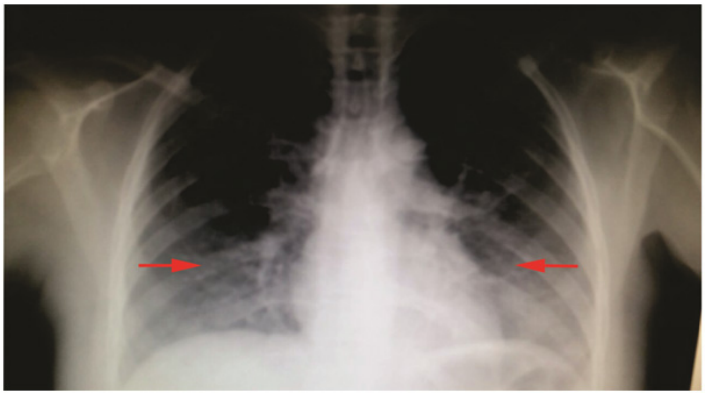

Figure 1: A chest X-ray showing bilateral interstitial pneumonia typical of *Pneumonia carinii pneumonia*.

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have periportum caesarean sections to salvage the fetuses. Fetal complications include iatrogenic prematurity,[16] stillbirths and congenital transplacental infection.[17-19]

Pneumocystis carinii pneumonia has a more aggressive course during pregnancy with increased morbidity and mortality, maternal[16] and fetal outcomes remaining dismal.[4]

CONCLUSION

Pneumocystis carinii pneumonia is a serious infection that causes significant maternal and fetal morbidity and mortality in HIV/AIDS patients. Widespread HIV testing and use of Co-trimoxazole chemoprophylaxis can reduce maternal and fetal morbidity and mortality. Co-trimoxazole is safe in pregnancy and breastfeeding. It must be a differential diagnosis of any pregnant HIV seropositive patient presenting with pulmonary symptomatology.

AUTHOR'S CONTRIBUTION

This is the sole work of Mr. S. Ngwenya.

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An Unbalanced Exon-Expression qPCR-based Assay for Detection of *ALK* Translocation (Fusion) in Lung Cancer

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ABSTRACT

Non-Small Cell Lung Cancer (NSCLC) constitutes 85-90% of all lung cancer. Accurate diagnosis and selection of targeted therapies in lung cancer depends on robust detection of the molecular events that underlie its pathogenesis. Since patients having a rearrangement in the Anaplastic Lymphoma Kinase (*ALK*) gene respond well to treatment with crizotinib, identification of such *ALK* mutations is necessary for the successful treatment of NSCLC. The most common rearrangement of the *ALK* gene in NSCLC involves fusion with echinoderm microtubule-associated protein-like 4 (EML 4) as the upstream partner. Current testing methods for this rearrangement (IHC and/or FISH) can be very subjective due to high operator variability. They require expert interpretation by a pathologist and have a long turnaround time. The FDA-approved Fluorescence *In Situ* Hybridization (FISH) test has been shown to lack sensitivity and is generally acknowledged to fail to detect rearrangements in up to 60% of patients. Here, we have adapted an approach described earlier and optimized it for use with degraded RNA obtained from Formalin-Fixed Paraffin-Embedded (FFPE) sections. This method is based on the unbalanced expression of 5'- and 3'-regions (exons) of the *ALK* gene. It is also applicable to the detection of other cancer-relevant gene rearrangements e.g. ROS 1 or RET that result in increased expression of the 3'-kinase domain. Patients with these rearrangements have been shown to respond to crizotinib and cabozantinib, respectively. Using NSCLC cell lines we demonstrate that our method is cost-effective, reproducible, sensitive, objective, and easy to use. Unlike FISH, it does not require interpretation by several scorers and it can be performed in any clinical laboratory with access to a qPCR instrument. Here we present the protocol for the method and validation with 197 clinical samples.

KEYWORDS: Non-small cell lung cancer; Targeted therapy; Unbalanced exon-expression; *EML4-ALK* rearrangement.

ABBREVIATIONS: NSCLC: Non-Small Cell Lung Cancer; PCR: Polymerase Chain Reaction; qRT-PCR: quantitative Reverse-Transcriptase-PCR; FFPE: Formalin-Fixed Paraffin Embedded.

INTRODUCTION

Lung cancer is the most common cancer and leading cause of death from cancer in Canada. Lung cancer represented 14% (26,600) of all new cancers diagnosed in Canada and accounted for 27% (20,900) deaths in 2015. This results in the death of 57 Canadians every day and the numbers will continue to rise with the aging population. Non-Small Cell Lung Cancer (NSCLC) constitutes 85-90% of all lung cancers.[1]

Therapeutic options for many cancers, including advanced lung cancer, are generally

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limited to chemotherapies, for which response rates are typically 20-30% with only modest increases (3-5 months) in Progression-Free Survival (PFS). In contrast, personalized therapy based on patients genotypes offer much higher response rates (approximately 75%), longer PFS (9-13 months) and fewer side effects. Between 3-11% of patients have activating mutations caused by various rearrangements in the Anaplastic Lymphoma Kinase (*ALK*) gene, which are targetable by the tyrosine kinase inhibitor, crizotinib (Pfizer, PF-02341066).[2] Additional second and third-generation ALK inhibitors are approved or in development such as ceritinib (Novartis), alectinib (Roche-GenenTech), lorlatinib (Pfizer), brigatinib (Ariad), entrectinib (Ignyta). Identifying patients with *ALK* rearrangements is currently expensive, time-consuming and subject to misinterpretation.[3]

Accurate diagnosis and selection of targeted therapies in lung cancer depends on robust detection of the molecular events that underlie its pathogenesis. It is known that patients having an activating mutation (rearrangement) in the *ALK* gene respond better to treatment with crizotinib (a tyrosine kinase inhibitor) than lung cancer patients with other etiologies of the disease. Therefore, identifying patients with *ALK* mutations becomes a prerequisite to successful and efficient treatment of lung cancer. The most common rearrangement of the *ALK* gene in NSCLC involves fusion with echinoderm microtubule-associated protein-like 4 (EML4) as the upstream partner.[4] There are many known variants of *EML4-ALK* fusions depending on which EML4-exon is fused with *ALK* exon 20.[4-6] For example, in the variant 1 EML4 exon 13 is fused with the ALK exon 20 (E13:A20) and in the variant 7 EML4 exon 14 is fused with *ALK* exon 20 (E14:A20). Other known upstream partner genes that are fused with the *ALK* gene in NSCLC are *TFG*,[7] *KIF5B*[8,9] and *KLC1*.[10] However, the existing methods that detect only specific, e.g. *EML4-ALK* translocations or those with known upstream fusion genes, are of limited scope.

The current testing methods for the *EML4-ALK* rearrangement are immunohistochemistry (IHC)[11-13] and/or Fluorescence *In Situ* Hybridization (FISH) using the Vysis Break Apart Probe.[14] These tests use a solid tissue biopsy and can be very subjective due to high operator variability. They require expert interpretation by a pathologist and have a long turnaround time (TAT). The ALK IHC test, which measures expression of protein, is only semi-quantitative. The staining intensity score (1-4) that is used to determine *ALK*-positive or negative samples,varies depending on the fusion points (*EML-ALK*) and the promotor strength of the upstream partner. An upstream partner of similar promotor strength as *ALK* would give a negative result by IHC but positive result by FISH. In contrast, epigenetic changes in the promotor region may also cause higher expression of an unrearranged *ALK* gene resulting in positive IHC but negative FISH. The FDA-approved FISH test has been shown to lack sensitivity and is generally acknowledged to fail to detect rearrangements in up to 60% of patients.[15,16] The FISH test also fails to identify cases where epigenetic change may cause upregulation of an unrearranged *ALK* gene.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) methods allow the detection of gene rearrangements using RNA extracted from a patient's tumor.[4-6] They have been shown to be more accurate in determining rearranged *ALK* compared to FISH,[17] enable rapid processing and are highly specific for particular rearrangements depending on the primers used, but will not detect unknown rearrangements. Here, we have adapted a method described by Wang et al[18] and optimized it for use with degraded RNA obtained from Formalin-Fixed Paraffin-Embedded (FFPE) sections. This method is based on the unbalanced expression of 5'- and 3'-regions (exons) of the *ALK* gene. In the normal unrearranged *ALK* gene, the expression levels of both 5'- and 3'- exons are equal, whereas in the rearranged-*ALK* gene, expression of the 3'-region (kinase domain) is higher than that of the 5'-region. It is also notable that *ALK*-positive patients identified by RT-PCR and Next Generation Sequencing show similar responses to crizotinib as those identified by FISH.[18,19]

The robust and quantitative RT-PCR (qRT-PCR) method we have developed can detect unbalanced 5'-and 3'-exon expression indicative of *ALK* rearrangement. Since the primers used to amplify the exons are not based on specific fusion-points, the test detects all known and unknown variants and all upstream fusion partners of *ALK* can be determined by sequencing amplicons if necessary. In addition, the test can also identify high *ALK*-expressers that may be caused by epigenetic changes or mutations in the promoter region. This unbalanced 5'-and 3'-exon expression approach is also applicable for detection of other cancer-relevant gene rearrangements that result in increased expression of the 3'-kinase domain, e.g. ROS1 or RET. This is particularly relevant to patients with these rearrangements as they have been shown to respond to crizotinib[20] and cabozantinib,[21] respectively. This method is reproducible, quantitative, sensitive (to 1% positive cells), requires only 50-100 ng RNA, does not require highly trained personnel and can be performed in any clinical laboratory with access to a qPCR instrument. Here we present the protocol for the method and validation with 197 clinical samples.

MATERIAL AND METHODS

Clinical Samples and Cell Lines

Fifteen FFPE slides containing 2×10 µm sections from Canadian *ALK* (CALK) centers were provided by Dr. Ming Tsao, Department of Pathology, University Health Network, Princess Margaret Cancer Center and University of Toronto, ON, Canada. A second set of 182 specimens of 20 µm curls from FFPE sections were obtained from our tissue bank at the Nova Scotia Health Authority and Dalhousie University, Halifax, NS, Canada.

The lung adenocarcinoma cell line A 549 harboring the normal unrearranged *ALK* gene was obtained from the American

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Type Culture Collection and propagated as recommended. The lung adenocarcinoma cell line H3122 harboring a rearranged *ALK* gene (EML4 exon13: *ALK* exon20; *ALK* Variant 1) was a kind gift from Dr. Jeffrey Engelmann, Massachusetts General Hospital, Boston, MA, USA. The lung adenocarcinoma cell line HCC78 carrying the SLC34A2 exon4: ROS1 exon 32 fusion was obtained from Dr. John D. Minna, The University of Texas Southwestern Medical Center, Dallas, TX, USA. This study was approved by the Capital Health Research Ethics Board (CDHA-RS/2013-090) and all participating individuals signed inform consent.

Primers and Stock Preparation

Primers were designed to span exon-exon boundaries to minimize amplification from contaminating genomic DNA (Table 1, Suppl. Table 1). Primers were obtained from IDT (Coralville, IA, USA) and resuspended in RNase-free water to a working stock concentration of 10 µM.

Total RNA Isolation

Tumor-containing tissue was scraped from FFPE slides (1×20 µm or 2×10 µm sections) into a 1.5 mL Eppendorf tube using a fresh scalpel blade treated with RNA-Zap (Ambion Inc., Austin, TX, USA). Deparaffinization was achieved by the addition of 1 mL xylene, vigorous vortexing for 10 s, and centrifugation at 14000 rpm for 2 min. The supernatant was carefully removed by pipetting without disturbing the pellet and the pellet washed with 1 mL 100% ethanol, vortexed, and centrifuged at 14000 rpm for 2-5 min. The supernatant was again removed by pipetting without disturbing the pellet and any residual ethanol was removed using a fine pipet tip. The tube was then incubated at room temperature (15-25 °C) or at 37 °C for 10 min or until all residual ethanol had evaporated. Care was taken that deparaffinization was complete and the tissue pellet was not over-dried preceding lysis.

Total RNA was extracted from FFPE sections using the RNeasy® FFPE Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instruction. Total RNA was eluted into 50 µL of RNase-free water. Total RNA from fresh cells was purified using the RNeasy® kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Human reference RNA was obtained from Life Technologies (Waltham, MA, USA). RNA concentration was determined using a NanoDrop-1000 (Nano Drop Technologies, Wilmington, DE, USA) and stored at -80 °C.

Reverse Transcription

Total RNA (500 ng), unless indicated otherwise, was reverse-transcribed using the Superscript III kit (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instruction. cDNA was used immediately for qPCR or stored frozen at -20°C.

RT-qPCR: qPCR reactions (10 µL) contained 5 µL 2X Kapa CybrFast qPCR mix (Kapa Biosystems, Boston, MA, USA), 10 pmol each of forward and reverse primers and 1 µL of undiluted cDNA. All reactions were performed in triplicate. The control cDNA was from the A549 cell line. The positive controls were

Primer	For/Rev	Sequence (5' > 3')	Amplicon Size (bp)
Assay optimization with cell lines			
ALK-Ex-1	For	GAGGCGATCTTGGAGGGTTG	154
ALK-Ex-1	Rev	CCACTTCCGACGCCTTCTTC	
ALK-Ex-29	For	AAAGAAGGAGCCACACGACAG	185
ALK-Ex-29	Rev	CGTAATTGACATTCCCACAAGG	
GAPDH 102-F	For	CAAGATCATCAGCAATGCCT	193
GAPDH 192-R	Rev	CTCTCCAGAACATCATCCCT	
Assay validation with clinical samples			
ALK14-15-FP	For	GTGAACAGAAGCGTGCATGAG	91
ALK14-15-RP	Rev	GCACCGGCACTCCATCCTTC	
ALK17-18-FP	For	CCACTGGGCATCCTGTACACC	112
ALK17-18-RP	Rev	CCATGTGACATTCGTCTACCTCAC	
ALK22-23-FP	For	CCTGAAGTGTGCTCTGAACAGG	87
ALK22-23-RP	Rev	GCGAACAATGTTCTGGTGGTTG	
GAP4-5-FP	For	GCCAAGGTCATCCATGACAAC	92
GAP4-5-RP	Rev	GGGCCATCCACAGTCTTCTG	

Table 1. List of primers used for *ALK* exon-expression assay.

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cDNA from the *ALK*-positive H3122 cell line or the ROS1-positive HCC78 cell line. The negative control was RNase-free water.

qPCR was performed in a Light Cycler® 480 Real-Time PCR System (Roche Applied Science, Laval, QC, Canada) in 384-well plates according to the Kapa CybrFast qPCR kit cycling conditions (once at 95 °C for 3 min; 45 times at 95 °C for 20 sec, 60 °C for 20 sec, 72 °C for 20 sec) followed by melt-curve analysis.

DATA ANALYSIS

The raw data was exported from the Light Cycler-480 software and an average Ct was calculated for each triplicate reaction. Fold-change was calculated using ΔΔCt method[22] as below:

ΔCt = Ct (Target A-Treated) – Ct (Ref B-Treated),
ΔCt = Ct (Target A-Control) – Ct (Ref B-Control),

Therefore, the fold-change for *ALK* exon14-15 using *GAPDH* as reference and A549 as control will be:

$\Delta\Delta Ct = \Delta Ct\ (ALK\ exon14\text{-}15^{FFPE} - GAPDH^{FFPE}) - \Delta Ct\ (ALK\ exon14\text{-}15^{A549} - GAPDH^{A549})$

The fold change = $2^{(-\Delta\Delta Ct)}$

The *ALK* 14-15 exon served as the normalizer. Fold-change for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) exon 4-5 was used to assess the level of degradation of the FFPE-derived RNAs compared to the cell line controls. For validation set 1, fold-change of the *ALK* exon 22-23 was determined after normalization with the *ALK* exon 14-15 to identify *ALK* rearrangement-positive or -negative samples. For highly degraded FFPE samples (validation set 2), normalization with *ALK* exon 14-15 and/or 17-18 gave inconsistent results. This was mainly because of RNA degradation. In an unrearranged ALK gene, normalized expression values decrease from the 5'- to the 3'-end. However, in a rearranged *ALK* gene, expression of the 5'-exon will be higher than that of the middle exon, but expression of the 3'-exon will be higher than the middle despite the effect of RNA degradation. Therefore, GAPDH exon 4-5-normalized expression of *ALK* exon 14-15, 17-18 and 22-23 was used to determine *ALK*-positives. A sample was called positive when *ALK* exon 22-23 expression was higher than the *ALK* exon 17-18, which is at the 5'-position to the fusion point (*ALK* exon 20). The identity of the upstream fusion partner is not known; only the presence of a rearrangement resulting in up-regulation of the 3' kinase domain of *ALK* is determined.

The utility of the exon-expression assay for the detection of the *ROS1* gene fusion was demonstrated using HCC78 cell line containing the SLC34A2: *ROS1* rearrangement. Primers designed for the *ROS1* 5'-end (exon 7 and 12) and *ROS1* 3'-end (exon 35 and 39) and GAPDH were used (Supplementary Table 1). Fold-change was derived as described above after normalization with *GAPDH*.

RESULTS AND DISCUSSION

As shown in Figure 1, expression of both 5'-exon 1 and 3'-exon 29 of intact *ALK* is similar and lower (higher Ct value; blue circle and arrow) compared to the 3'-exon 29 in a rearranged *ALK* (red arrows). The two groups of amplification curves in both groups are due to undiluted and 10-fold diluted starting template cDNA

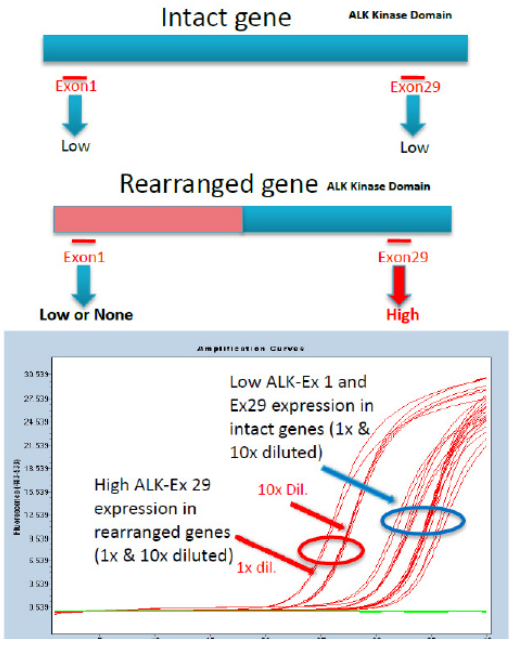

Figure 1: *ALK* exon-expression assay for rearrangements. Amplification curves are shown for RNA isolated from cell lines containing unarranged and rearranged *ALK* genes as described in Material and Methods.

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used in the qPCR reactions. Unbalanced expression is observed when the *ALK* gene is rearranged irrespective of the rearrangement partner or fusion points in the case of *EML4-ALK* variants. Therefore, the greatest advantage of this method is the ability to detect not only known but also unknown *EML4-ALK* variants as well as fusion with other known and unknown upstream partners (e.g. KIF5B, TFG, KLC1), which upon further study may prove sensitive to crizotinib. Identification of fusion points and upstream partner can be determined, if desired, by qPCR of each *ALK*-exon and sequencing of the amplicon containing the fusion partner.

To determine the sensitivity of detection of the test, a titration was performed with the known *EML4-ALK* rearranged cell line H3122 into the A549 cell line containing the unrearranged *ALK* (Figure 2). *ALK* unbalanced 3'-exon29 expression can be detected with high confidence (*p*-value 0.0025) with as few as 1% H3122 cells in A549 background. In contrast, since tumors are heterogeneous, FISH-positive cell count variations are inherent.[23] For example, 5-95% FISH-positive cells were scored for the sample CALK-FFPE-11 by 2 individual operators at twelve centers. The accepted cutoff value is 15% FISH positive cells. This sample was confirmed negative for *ALK* rear-

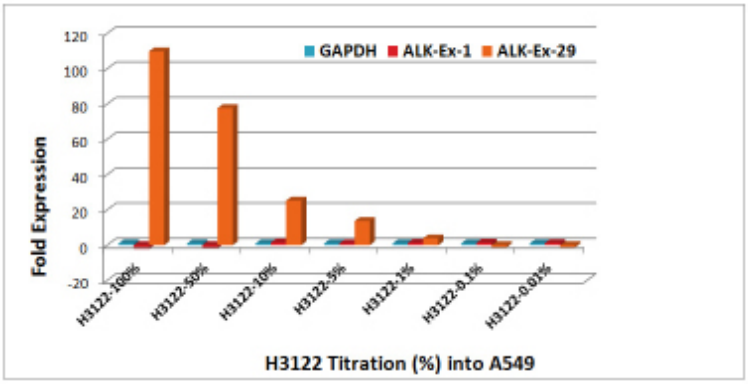

Figure 2: Determination of sensitivity of detection of rearranged ALK gene by dilution. H3122 RNA (EML13:*ALK*20) was diluted into A549 (intact ALK). All p-values for assays down to 1% H3122 RNA were 0-0.0025.

	ALK Exon-Expression qRT-PCR					FISH*	
Sample Name	GAPDH		*ALK*- 3'-exon		Positive / Negative	FISH- % Positive cells[a]	Positive / Negative
	Fold Change	*p* value	Fold Change	*p* value			
H3122 (Pos. Ctr.)	-1.32	0.009	219.79	0.0000	Positive	N/A	N/A
CALK-FFPE-1	-49.52	0.00029	14.12	0.015	Positive	50-99	Positive
CALK-FFPE-4	-134.67	0.00002	4.42	0.000328	Positive	25-70	Positive
CALK-FFPE-7	-29.31	0.000028	4.84	0.00007	Positive	15-80	Positive
CALK-FFPE-8[b]	-105.17	0.000025	2.25	0.0381	Positive	24-70	Positive
CALK-FFPE-9	-27.22	0.00002	2.54	0.236	Negative	0-5	Negative
CALK-FFPE-10	-171.65	0.000025	2.8	0.0053	Positive	5-75	Positive
CALK-FFPE-11	-15.17	0.000047	1.92	0.1427	Negative	5-95	Negative
CALK-FFPE-12	-2.68	0.00165	240.51	0.000004	Positive	25-90	Positive
CALK-FFPE-13	-130.99	0.000025	1.45	0.19844	Negative	5-12	Negative
CALK-FFPE-14	-30.69	0.000028	1.47	0.126358	Negative	5-10	Negative
CALK-FFPE-15[c]	-434.54	0.000024	-1.25	0.22542	Negative	10-60	Positive
CALK-FFPE-17	-88.85	0.000025	14.16	0.000051	Positive	20-90	Positive
CALK-FFPE-19	-33.2051	0.000304	58.35	0.000005	Positive	30-90	Positive
CALK-FFPE-23	-168.89	0.000025	2.32	0.077846	Negative	5-10	Negative
CALK-FFPE-27	-123.06	0.000025	5.72	0.000759	Positive	15-60	Positive
CDHA-A4[d]	-23.16	0.00032	16.45	0.000212	Positive	N/A	N/A
CDHA-C-1[d]	-6.96	0.000491	43.31	0.0023	Positive	N/A	N/A

Note: A549 Negative control and reference sample; *H3122-ALK* Positive control sample
a: Distribution of % abnormal *ALK* signals from 200 nuclei counted by 2 technologist from 12 Centers
b: only 160ng RNA available for RT
c: FFPE section was very bloody; GAPDH >400-fold degraded
d: Known Positive FFPE from Dr Zhaolin Xu
*: From Reference[17]

Table 2: Validation of *ALK* exon expression assay using clinical FFPE samples from *CALK* centers. Fold change of GAPDH represents the amount of RNA degradation compared to H3122 cell line RNA, which is relatively intact (-1.32). Fold change of *ALK* represents the expression of the 3'-exon22-23 relative to the 5'-exon17-18 after normalization with *ALK* exon14-15 as described in the Materials and Methods. *ALK* positive tumors have a fold change of greater than 2 with a *p*-value <0.05. N/A, FISH test was not performed.

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rangement using RT-PCR method (Table 2). However, in RNA extracted from a 10-20 µm FFPE section, all RNA species are available for detection by qPCR, and RNA contributed by as little as 1% *ALK*-positive cells is detected.

Due to the limited availability of patients' FFPE samples together with limited amounts of total RNA available after purification, a titration experiment was performed to determine the minimal amounts of RNA required for cDNA conversion prior to qPCR. Most reverse transcription kit protocols suggest using 500-1000 ng total RNA for cDNA synthesis. We converted 10-500 ng total RNA into cDNA and evaluated unbalanced exon-expression using H3122 and HCC78 cell lines harboring rearranged *ALK* and *ROS1*, respectively (Figure 3). Total RNA amounts from 10-500 ng showed >50-fold higher 3'-end expression compared to the 5'-end with very high confidence (*p*-value 0.000001). However, total amounts of 20-100 ng RNA converted into cDNA provided optimal performance. This demonstrates an additional advantage of this method where a single 10 µm FFPE section or a very small biopsy sample may be sufficient to determine rearranged *ALK* and/or *ROS1* gene.

The ROS1 5'-end exons (7 and 9) have a higher Ct value (lower expression) compared to ROS1 3'-end exons (35 and 39) in HCC78 cell line (Supplementary Figure 1). As expected, expression of the housekeeping gene *GAPDH* is similar in the A549 cell line containing intact *ROS1* gene and *HCC78*. The sensitivity of the *ROS1* exon-expression assay was determined by titration of *HCC78* RNA into A549 RNA (Supplementary Figure 2). As little as 1% *HCC78* RNA in A549 RNA was detectable (*p*-value 0.000001). Validation of the *ROS1* exon-expression assay was not performed due to unavailability of clinical samples.

The *ALK* exon-expression assay was validated in a blinded manner using 15 FFPE samples from NSCLC patients from *CALK* centers (Validation set 1). Two known positive FFPE samples were also included. We were unable to obtain amplification using *ALK* exon1 and *ALK* exon29 and *GAPDH* 102F and 192 R primers (Table 1), indicating that RNA from FFPE samples was degraded to sizes smaller than these amplicons (154-193 bp). To address this, new exon-exon spanning primers for *ALK* exons 14-15, 17-18 and 22-23 were designed that generated smaller amplicons (87-112 bp; Table 1). The *ALK* exon 14-15 was used to normalize and determine relative expression of *ALK* exon 17-18 and *ALK* exon 22-23 and also to determine the amount of RNA degradation in FFPE samples compared to cell line RNA. H3122 RNA exhibited no degradation (fold-change of -1) whereas FFPE RNAs showed varying amounts of degradation (Table 2). Samples that had >2-fold higher expression of 3'-exon 22-23 and a *p*-value 0.05 or lower were scored as *ALK*-positive. Of the 15 CALK samples, 14 were concordant with FISH results (Table 2). *CALK*-FFPE-11 was *ALK*-negative using the exon-expression assay. Interestingly, this sample showed very high variability by FISH (5-95% cells FISH-positive as scored by two technologists at 12 centers) but was called negative when subsequently tested by RT-PCR[17] in agreement with our results (Table 2). Similar heterogeneity in FISH results has been reported previously.[23] *CALK*-FFPE-15 exon-expression assay results were not concordant with the FISH results; this is probably because the FFPE section for this sample was very bloody and also showed a very high level of RNA degradation (*GAPDH* fold-change; -434-fold), making it impossible to assess with certainty.

A second set of 182 validation samples were single 20 µM curls (Validation set 2). Good concordance was seen between the exon-expression assay and FISH. Similar to Validation set 1, A549 and H3122 cells were used as negative and positive controls and relative *GAPDH* expression was used to assess RNA degradation in the FFPE samples. Table 3 shows the results of six samples that had equivocal results. These samples showed varying and high levels of RNA degradation when compared with cell line RNA (Table 3). When RNA is highly degraded, the accuracy to assess fold-changes between 5'-end and 3'-end expression by ΔΔCt method is compromised. However, the test still yields a result and can be confirmed with IHC and/or FISH. Degradation of any transcript starts from the 3'-end and proceeds towards the 5'-end which is capped to prevent degradation. Sample RE-12-04 T RNA showed RNA degrada-

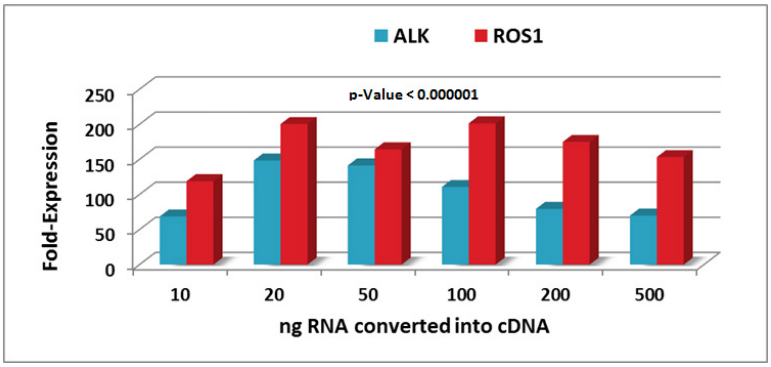

Figure 3: Determination of sensitivity of detection of rearranged *ALK* and ROS1 genes. Decreasing amounts of total RNA were used in the assay as described in the Materials and Methods.

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Sample Name	FISH						*ALK* Exon-Expression by qRT-PCR (*ALK* exon14-15 and 17-18 normalizer)						*ALK* Exon-Expression by qRT-PCR (*GAPDH* exon4-5 normalizer)							
	% Abnormal cells					FISH results -NSHA	*GAPDH* Exon 4-5		*ALK* Exon 22-23		Result qPCR method	Concordance with FISH	*ALK* Exon 14-15		*ALK* Exon 17-18		*ALK* Exon 22-23		Result qPCR method	Concordance with FISH
	Read 1	Read 2	Read 3	Read 4	Read 5		Fold-Change	*p* value	Fold-Change	*p* value			Fold-Change	*p* value	Fold-Change	*p* value	Fold-Change	*p* value		
RE-12-04 T	48%	74%	NA	NA	NA	Pos.	-513.00	0.0008	-1.01	0.8963	Neg.	NO	299.55	0.0003	156.50	0.0170	284.05	0.0154	Pos.	YES
RE-12-04 AB	56%	80%	NA	NA	NA	Pos.	-185.00	0.0013	2.29	0.0900	Neg.	NO	185.25	0.0000	99.96	0.0000	312.27	0.0169	Pos.	YES
RE-12-04 BL	9%	14%	1%	34%	NA	Neg.	-2.50	0.0311	3.54	0.0178	Pos.	NO	2.50	0.0124	-2.85	0.0003	3.33	0.0064	Pos.	NO
RE-12-04 W	8%	4%	9%	16%	NA	Neg.	-316.00	0.0008	2.68	0.0130	Pos.	NO	135.92	0.0310	41.45	0.2410	54.06	0.0012	Pos.	NO
RE-12-04 BV	41%	36%	4%	6%	26%	Neg.[a]	-3.56	0.0035	2.02	0.0195	Pos.	NO	9.36	0.0017	1.33	0.0074	7.11	0.0005	Pos.	NO
RE-12-05 AU	15%	18%	8%	8%	12%	Neg.[a]	-72.58	0.0010	1.56	0.4207	Neg.	NO	74.88	0.0461	70.36	0.0080	113.50	0.0042	Pos.	NO

[a]Additional confirmatory FISH test performed at *CALK* center Toronto. This was also scored as Negative.

Table 3: Results of *ALK* exon-expression assay for Validation set 2 samples. Fold change of GAPDH exon4-5 represents the amount of RNA degradation compared to H3122 cell line RNA, which is relatively intact (-1.32). *ALK*-positive tumors have a fold change >2 with a *p*-value <0.05 (shown in red). For GAPDH exon4-5 normalized data, *ALK* positive tumors have higher fold change for *ALK* 3'-exon22-23 relative to *ALK* 5'-exon17-18 with a *p*-value <0.05 (shown in red). qPCR results are scored positive (Pos.) or Negative (Neg.) and concordance with FISH result is scored YES or NO.

tion of >500-fold and did not clearly show >2-fold change with high confidence when normalized with *ALK* exon 14-15/ 17-18. However, *GAPDH*-normalized expression values (284 for exon 22-23 compared to 156 for exon 17-18) indicated that this sample is *ALK*-positive (*p*-value<0.05; Table 3). Similarly, sample RE-12-04 AB showed ~185-fold degradation of RNA and only 2.3-fold higher expression of 3'-end ALK with *p*-value 0.09 when normalized with *ALK* exon14-15/17-18. Again, GAPDH-normalized expression (312 for exon 22-23 compared to 100 for exon 17-18) suggests this sample is *ALK*-positive (*p*-value <0.01; Table 3). Both RE-12-04 T and RE-12-04 AB samples were *ALK*-positive by the FISH test with 48-74% and 56-80% abnormal cells, respectively (Table 3).

Sample RE-12-04-BL was positive by exon-expression assay using both normalization methods (Table 3). This sample was negative by FISH even though average of the 4 reads is ~15% abnormal cells. Two other samples, RE-12-04-W and RE-12-04-BV were also positive with both normalization methods. However, these samples were negative by the FISH with positive cell counts of 9±5% and 23±16% (average±standard deviation), respectively (Table 3). Sample RE-12-05-AU was found positive by using *GAPDH* as normalizer (113.5 for exon 22-23 compared to 70.36 for exon 17-18) but not when exon 14-15/17-18 was used. This sample was negative by the FISH test with 12±5% counted as FISH-positive (Table 3). It is possible that an epigenetic modification of the *ALK* gene or a rearrangement that alters FISH probe binding would not be detected by FISH and caused relative up-regulation of exon 22-23 in this tumor.

Clearly, the sensitivity of the assay depends on the quality of the RNA. As seen for the validation samples, high levels of RNA degradation can lead to a decrease in the ability to determine *ALK*-positivity by the exon-expression assay. Therefore, we compared the RNA quality from ten representative FFPE samples and RNA isolated from a fresh-frozen lung tumor sample and a commercially available human reference RNA. We used a qPCR method measuring 93bp and 193bp amplicons of the highly expressed housekeeping gene, *GAPDH*. Average Ct values of three replicates for both amplicons are shown in Suppl. Table 2. The Ct values for both amplicons from fresh-frozen lung tumor and reference human RNA are similar and lower, indicating little or no degradation of RNA, whereas RNA from all FFPE samples showed 10-15 Ct value higher or not even detectable (Ct>35). This translates into 1000-100,000-fold degradation of RNA obtained from FFPE samples compared to fresh-frozen samples. Such samples may necessitate confirmation with another method.

CONCLUSION

The qPCR-based exon-expression assay we have developed is demonstrably objective, robust and sensitive for the detection of rearranged *ALK* genes when compared to FISH and IHC. As shown by our preliminary results with *ROS1*, this method also shows great promise as generalized test for rearranged genes implicated in various kinds of cancer. This is particularly relevant since *ROS1*-positive patients also respond to crizotinib. Since detection of rearranged genes in patient samples uses RNA, the sensitivity and robustness of this test is compromised when RNA is highly degraded, and can result in false-negative calls. We have observed this phenomenon with two sets of valida-

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tion FFPE samples showing high degrees of RNA degradation. However, fresh patient samples may have less RNA degradation, which would minimize false-negative calls; therefore, we highly recommend using fresh-frozen or RNA Later-preserved biopsies for this assay.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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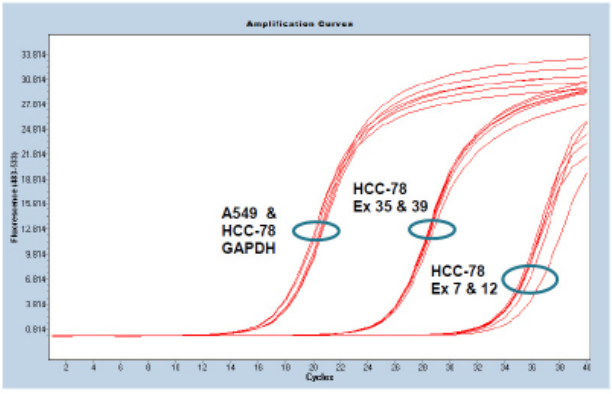
Supplementary Data

Primer	For/Rev	Sequence (5' > 3')	Amplicon Size
GAPDH 102-F	For	CAAGATCATCAGCAATGCCT	193bp
GAPDH 192-R	Rev	CTCTCCAGAACATCATCCCT	
ROS1-Ex-7	For	TGAGAGCTCAAGTCCCGACAC	130bp
ROS1-Ex-7	Rev	GGTTCTCTGTGTCCCTGCATC	
ROS1-Ex-12	For	CTGTGCGTATTGTGGAGAGTTG	137bp
ROS1-Ex-12	Rev	TAGGATGAGATGGGAAGCAGAG	
ROS1-Ex-35	For	ATTGAAAATCTTCCTGCCTTCC	127bp
ROS1-Ex-35	Rev	TGATTTCTCCACTTCCAACTCC	
ROS1-Ex-39	For	CAGCTAGAAATTGCCTTGTTTCC	125bp
ROS1-Ex-39	Rev	CAGGCCTTCCCCTCTCTTTC	

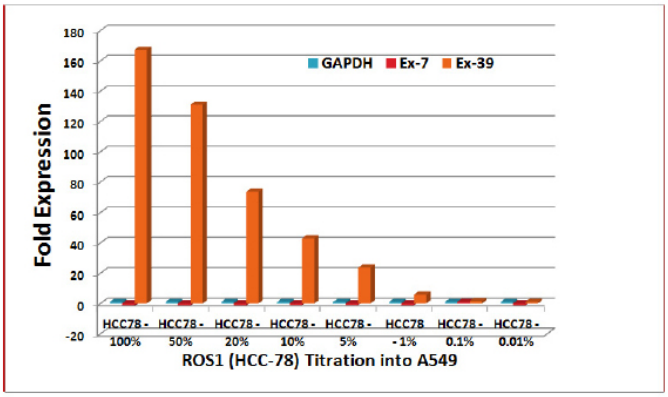
Supplementary Table 1: List of primers used for *ROS1* exon expression assay.

Sample Name	**GAPDH: Average Ct Value** (3 replicates)	
	93 bp amplicon	**193 bp** amplicon
Lung-FFPE 1	28.88	33.37
Lung-FFPE 2	27.17	29.93
Lung-FFPE 3	30.02	>35.00
Lung-FFPE 4	29.46	>35.00
Lung-FFPE 5	28.60	32.49
Lung-FFPE 6	25.65	27.91
Lung-FFPE 7	27.55	31.15
Lung-FFPE 8	26.51	28.52
Lung-FFPE 9	27.35	29.79
Lung-FFPE 10	27.55	30.34
Fresh-Frozen Lung Tumor	16.89	15.98
Human Reference RNA	16.30	14.74

Supplementary Table 2: Comparison of RNA quality between RNA isolated from FFPE samples and a fresh-frozen sample by qRT-PCR. RNA was extracted from ten FFPE samples and one fresh-frozen sample and compared with Human Reference RNA by qPCR as described in the Materials and Methods.



Supplementary Figure 1: Detection of *ROS1* fusion by exon-expression assay. RNA was extracted from the HCC-78 cell line carrying SLC34A2-ROS1 fusion (SLC34A2 Ex4:*ROS1* Ex 32). A549 ROS1 exon 7, 12, 35 and 39 amplify similar as HCC-78 ROS1 exon 7 and 12 (not shown).



Supplementary Figure 2: Determination of sensitivity of detection of translocated *ROS1* by dilution of HCC-78 RNA (SLC34A2 Ex4:*ROS1* Ex 32) into A549 RNA (intact *ROS1*). All *p*-Values for assays down to 1% HCC-78 RNA were 0-0.000001.

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Commentary

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Modern Technology in Respiratory Medicine: Lung Ultrasonography–Is it Time for the Stethoscope to Give Up its Throne?

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The year 2016 marks the 200th anniversary of the invention of the stethoscope by Dr. Réne Théophile Hyacinthe Laennec. He was first inspired in September 1816 when he observed 2 children playing with a long piece of solid wood and a pin. He later made the very first stethoscope when he rolled a paper to listen to the heart sounds of a young woman. He further refined the instrument by constructing it with a hollow tube of wood.[1] Three years later, in 1819, he published a textbook, which has been the foundation of respiratory medicine. In his textbook titled "*De l'Auscultation Médiate ou Traité du Diagnostic des Maladies des Poumonset du Coeur (On Mediate Auscultation or Treatise on the Diagnosis of the Diseases of the Lungs and Heart)*", he introduced the terms that we still use in respiratory medicine, such as "rale", "rhonchi," or "egophony". Laennec founded the basis of modern respiratory medicine with the invention of this remarkable tool. The stethoscope remains an indispensable tool for physicians, and not a single day passes without it being used to examine a patient. However, in the last decade, notable controversies have emerged regarding the utilization of the stethoscope.[2]

It is interesting to learn how this new technology was perceived when Laennec first introduced the stethoscope. While this diagnostic tool was generally well received, as reported by the New England Journal of Medicine in 1821,[3] Simmons described in his book that some physicians rejected this instrument in the 20th century and preferred to apply their ears to their patients.[4]

The history of lung ultrasonography, on the other hand, is relatively new. Lichtenstein published a paper in 1995 that assessed movement of the lung surface to demonstrate the absence of pneumothorax, which has been termed as "lung sliding".[5] He also proposed to assess lung parenchyma itself by utilizing artifacts, such as reverberation artifact and ring-down artifact, which he named A-line and B-line.[6] Given its relatively new history, it may not be surprising that the utilization of lung ultrasonography has not been fully appreciated in the field of internal medicine.[7] Lung ultrasonography is unique in the utilization of artifacts that were often considered to impede the analysis of images.

Diaphragm ultrasonography is another example of a technology that has changed the diagnostic approach to assess diaphragmatic function. Before this imaging modality was developed, it was relatively time consuming and invasive to diagnose diaphragm dysfunction by fluoroscopy or by electromyography. McCool et al[8] reported that with the aid of ultrasonography, the movement of the diaphragm dome and thickening of the diaphragm itself may be directly observed in real time. This can be performed at the bedside in the intensive care unit (ICU) or in the clinic for convenient identification of the presence or absence of diaphragm dysfunction. Though its history is relatively new, ultrasonography is becoming a standard tool in the field of

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pulmonary and critical care medicine. Mayo et al[9] at the American College of Chest Physicians (ACCP) have already recognized its importance and published a statement in competencies of ultrasonography for critical care physicians 7 years ago.

Regarding diagnostic accuracy, the stethoscope may not be as strong a tool as hoped for, especially for trainees. A study by Mangione revealed a striking deficiency in pulmonary auscultatory skills among internal medicine and family practice trainees as they recognized less than half of all respiratory events.[10] He also described "disturbingly low" cardiac auscultatory skills among internal medicine and family medicine trainees since they recognized 20% of all cardiac events.[11] Lung ultrasonography, on the other hand, clearly gives different results. Lichtenstein described in his original article that the absence of lung sliding to detect pneumothorax has extremely high sensitivity of 95.3% and specificity of 91.1% with positive predictive value of 87% and negative predictive value of 100%.[5]

People may have a reasonable concern regarding new technology, as the novelty of using such technology may distract physicians from focusing on their patients. Criticism is often directed towards physicians who tend to care less about the crucial part of data gathering by history and physical examination. While we acknowledge these potential pitfalls of new technologies, we should not deny its potential to revolutionize the world of medicine, as seen with the stethoscope, chest radiograph, or computed tomography, and the potential to create an entire new horizon, as Laennec did with the stethoscope 200 years ago.

In conclusion, the time for the stethoscope to give up its throne to lung ultrasonography may not have arrived; although in the near future, such an occurrence may be likely as the use of lung ultrasonography increases in the world of respiratory medicine. Hopefully, it will follow the path of its predecessor to revolutionize the field of medicine.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Mini Review

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Pleural Diseases in Pregnancy: Aetiology and Management

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ABSTRACT

Pleural diseases in pregnancy can cause considerable maternal and fetal morbidity and mortality. Some like pleural effusions may be small and pose like danger or massive causing significant respiratory compromise. Others like pneumomediastinum are rare in pregnancy but can be fatal. Emergency interventions may save lives. Such interventions may include periportum caesarean section. The management of such conditions calls for the involvement of a multi-disciplinary team. Clinicians caring for pregnant women must always be well prepared to deal with any respiratory emergencies that may arise and be prepared to take prompt and bold decisions to give life-saving treatment.

KEY WORDS: Pleural diseases; Pleural effusions; Pneumothorax; Haemothorax; Periportum caesarean section.

INTRODUCTION

Pleural diseases in pregnancy can cause significant maternal and fetal morbidity and mortality. It is important that clinicians caring for pregnant women are well informed about these conditions so that they make early diagnosis and institute prompt treatment plans. Pleural effusions, pneumomediastium and pneumothorax are known complications of pregnancy.[1] The other pleural conditions that can occur in pregnancy are empyema that can occur after a pneumonic spell and haemothorax[2] following a ruptured ectopic pregnancy. Pregnancy is a risk factor for pulmonary complications due to its immunosuppressive nature. Pulmonary symptomatology may be confused with normal physiological changes. Clinicians should maintain vigilance to differentiate pathology from normal physiology. Areas with a high prevalence of HIV/AIDS have high incidences of pleural diseases. HIV and pulmonary tuberculosis in pregnancy lead to a high chance of pleural disease complications. Careful multi-disciplinary team management involving the obstetricians, paediatricians, anaesthetists, pneumologists and thoracic surgeons in intensive care settings can save lives.

AETIOLOGY

Pregnancy complicated by hyperemesis gravidurum can result in oesophageal perforation resulting in pneumomediastinum[2,3] and pleural effusions.[4,5] *In vitro* fertilisation techniques can complicate with severe ovarian hyperstimulation system resulting in pleural effusions.[6] Metastatic disease may present with pleural effusions. Pulmonary tuberculosis can complicate with bilateral pleural effusions[7] as well as severe preeclamptic patients may also complicate with pleural effusions.[8] Spontaneous pneumothoraces can occur in pregnancy[9-11] and these may be recurrent.[12]

CLINICAL PRESENTATION

The signs and symptoms may include dyspnoea, cough and chest pains. In pregnancy chest

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symptomatology may be confused with normal physiology of pregnancy. Some patients may be asymptomatic. Clinical examination may reveal fever, tachycardia, tachypnoea and central cyanosis. Those patients with serious conditions may present with altered levels of consciousness. There could be dullness or resonance on chest percussion. On auscultation, there may be reduced or no air entry or coarse crepitations depending whether there is fluid or air in the pleural space. In cases of pneumomediastium there could be subcutaneous emphysema in the chest and neck. Some patients may present with respiratory distress with collapse.

INVESTIGATIONS

Arterial blood gases may be normal or reveal hypoxemia and metabolic acidosis. A chest X-ray would be diagnostic in most of the diseases showing pneumothoraces, pleural effusions and pneumomediastinum. Ultrasography can detect pleural diseases such as pleural effusions, empyema or haemathoraces. Specimens obtained from ultrasound guided pleural aspiration/drainage must be sent for cytological, histological and microbiological assessments including tests for acid fast bacilli.

A computed tomography (CT) may reveal more information about the lesion showing fluid (pleural effusion) or air (pneumothorax) or septations (empyema). Magnetic resonance imaging (MRI) is now increasingly being used for assessment of lung conditions such as metastasis, lymphoma, lipoma, endometriosis and empyema.[13] It gives better clinical information on the extent of the disease and its relation to surrounding tissue structures.

MANAGEMENT

Interventional pulmonology encompasses pleural interventions.[14] Conservative management of small pleural effusions may be appropriate. Empyema may be initially managed conservatively with antibiotics. Small tuberculosis pleural effusions may resolve with anti-tuberculosis chemotherapy. If medical treatment fails or the patient's condition deteriorates, surgical interventions would be appropriate. Patients with oesophageal perforation need emergency primary repair.[5] Those patients with a ruptured ectopic pregnancy would need an emergency laparotomy. In pregnancy, pneumomediastium is a rare condition but could be rapidly fatal hence urgent surgical intervention is needed. Urgent thoracostomy for patients in respiratory distress relieves pneumothoraces and haemothoraces while awaiting definitive treatment.[10,15] Pleurodeisis could cause infection, lung punch and fibrosis.

Thoracoscopic[16] treatment can be carried out such as video-assisted thoracic surgery for the treatment of empyema.[17] In cases of collapsed pregnant patients, a periportum caesarean section may help deliver a live infant or help with the resuscitation of the mother and improving her chances of survival. The fetus may be premature and suffer complications associated with prematurity such as respiratory distress syndrome and may demise. Clinicians should be prepared to do this procedure as it could be life-saving. Patients may need intensive care management with ventilatory support.

Repeat imaging may be necessary to check resolution/recurrence of the condition. During the course of treatment it is important to continue to monitor the fetus with ultrasound scans/cardiotocograms depending on the gestational stage. Serial growth scans would also be appropriate. The fetus may complicate with intrauterine growth restriction or intrauterine death if there is profound and prolonged maternal hypoxia. There is a risk of premature labour. Delivery would be by vaginally or by caesarean section depending on the severity of maternal health and obstetric factors.

PROGNOSIS

The prognosis is good[9] for both the fetus and the mother provided the pulmonary disease is well treated and no further complications occur.

CONCLUSION

Pleural diseases in pregnancy may threaten maternal and fetal lives. However, if the conditions are well managed by multi-disciplinary teams the outcomes may be favourable. It is incumbent upon clinicians caring for pregnant women to be alert to distinguish between pathology and normal physiology in pregnancy so that pregnant patients receive appropriate timely interventions. Repeat clinical examinations and imaging are needed to check for disease resolution or recurrence.

AUTHOR CONTRIBUTION

This is the sole work of Mr. S. Ngwenya

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