ASSOCIATION OF CT THORAX FINDINGS WITH GALACTOMANNAN AND *ASPERGILLUS* - SPECIFIC POLYMERASE CHAIN REACTION FOR DIAGNOSIS OF INVASIVE ASPERGILLOSIS IN PATIENT WITH FEBRILE NEUTROPENIA

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For my beloved husband and children, thank you so much for the understanding and unconditional support in going through the hard moments in complete this study. Last but not least, many thanks to all lecturer and colleagues in department of Radiology HUSM whom direct or indirectly involved in this study.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

IPA	Invasive Pulmonary Aspergillosis
EORTC-MSG	European Organization for Research and Treatment of Cancer/Mycosis Study Group
PCR	*Aspergillus*-specific Polymerase Chain Reaction
GM	Galactomannan
CT	Computed Tomography
HRCT	High resolution computed tomography
ANC	Absolute neutrophil count
OD	Optical Density

ABSTRAK

Latar belakang: *Computed* tomografi (CT) mungkin menunjukkan ciri-ciri tertentu Invasif Pulmonari Aspergillosis (IPA). Ciri-ciri ini termasuk A*irway* invasif dan *Angio* Invasif. Walau bagaimanapun kedua-dua jenis penemuan CT ini tidak khusus untuk IPA dan diagnosis mesti berdasarkan EORTC-MSG. Tujuan kajian ini adalah mencari perkaitan di antara ciri CT dada dengan Galactomannan(GM) dan *Aspergillus*-spesifik polymerase chain reaction(PCR) bagi pesakit yang mengalami *febrile neutropenia*.

Kaedah: Kesemua pesakit kanser darah yang mengalami *febrile neutropenia* 3 hari atau lebih walaupun telah dirawat dengan antibiotik akan menjalani ujian CT dada dalam masa 2 minggu. Penemuan CT dada akan direkod dan perkaitan antara jenis penemuan CT dan GM serta PCR dikaji menggunakan 'Fisher exact test'. (Nilai p signifikan <0.05).

Keputusan: Seramai 26 pesakit telah dimasukkan dalam kajian ; 17 (65.4%) CT dada adalah positif , di mana 8 (30.8%) positif untuk Galactomannan dan 9 (34.6%) untuk PCR. Terdapat hubungan yang ketara antara penemuan PCR dan CT dada (p value 0.009, 95% CI) tetapi tidak antara Galactomannan dan CT dada (nilai p 0.667, 95% CI).

Kesimpulan: Kombinasi di antara PCR dan CT dada boleh digunakan sebagai salah satu cara *noninvasive* untuk mengesan pesakit IPA dengan seawal mungkin. *Aspergillus* -spesifik PCR mungkin boleh dimasukkan sebagai salah satu kriteria *mycology* dalam EORTC-MSG untuk mengesan IPA pada masa akan datang.

Kata Kunci*: Invasive Pulmonary Aspergillosis, galactomannan, Polymerase chain*

reaction,Radiography thoracic, febrile neutropenia

ABSTRACT

Background: The Computed Tomography (CT) features of Invasive Pulmonary Aspergillosis (IPA) include Airway and Angio Invasive type. However those features are nonspecific and diagnosis should be made in conjunction with EORTC-MSG criteria. In this study, we evaluated the association between the CT findings and Galactomannan and *Aspergillus* specific PCR in patient with febrile neutropenia.

Methods: Patients with hematological malignancy who had persistent febrile neutropenia of 3 days or more despite of antibiotic underwent CT thorax within 2 weeks of clinical diagnosis of IPA. Blood serum for Galactomannan assay and *Aspergillus* specific PCR were performed. Changes of CT thorax were documented and association between mycological findings were analyzed using Fisher exact test. Significant association taken at p value of < 0.05.

Results: A total of 26 patients were enrolled in our study. CT findings were positive in 17(65.4%), 8(30.8%) was positive for Galactomannan and 9(34.6%) for PCR. There was significant association between PCR and CT thorax finding (p value 0.009, 95% CI) but not between Galactomannan and CT Thorax (p value 0.667, 95% CI).

Conclusion: Combination of PCR and CT thorax can be used as a non-invasive tools for early diagnosis of IPA. The *Aspergillus* specific PCR might to be consider as one of the mycological criteria in EORTC-MSG for diagnosis of IPA in the future.

Keywords: *Invasive Pulmonary Aspergillosis, galactomannan, Polymerase chain reaction, Radiography thoracic, febrile neutropenia*

CHAPTER 1 : INTRODUCTION & LITERATURE REVIEW

1.1 INTRODUCTION & LITERATURE REVIEW

Pulmonary Aspergillosis is caused by *Aspergillus fumigatus* lung infection. There are four manifestations of *Aspergillus* lung infection which is include Pulmonary Aspergilloma, Allergic Bronchopulmonary Aspergillosis, Chronic Necrotizing or Semi Invasive Aspergillosis and Invasive Aspergillosis[1]. The manifestations of *Aspergillus* lung infection depends on an individual immune response.

Aspergilloma is a colonization of a pre-existing lung cavity with little invasion of adjacent lung in normal individual immune status. Usually most of the cavities are due to Tuberculosis or Sarcodoisis [2]. Whereas Allergic Bronchopulmonary Aspergillosis (ABPA) is due to hypersensitivity reaction within the airways secondary to *Aspergillus* infection leading to wall damage, bronchiectasis and fibrosis. It is common in patient with poorly controlled asthma and cystic fibrosis[3]. Typically, there is elevation of serum IgE and positive skin test. The other spectrum is Chronic Necrotizing or Semi Invasive Aspergillosis. It demonstrate a more chronic disease course that seen along with Invasive Aspergillosis and can occur in patient with normal or mildly impaired immune system. Usually the treatment require long term antifungal and surgical might be considered [4].

The most important spectrum in *Aspergillus* infection is Invasive Pulmonary Aspergillosis (IPA) where it affects severely immunocompromised hosts. It first described by Rankin and was published in1953. In early course of disease, it will affect the airway (Airway Invasive Aspergillosis) before further vascular invasion (Angio Invasive Aspergillosis) causing necrosis of the lung parenchyma. Nowadays, Invasive

Pulmonary Aspergillosis still remain an important cause of morbidity and mortality among neutropenic patient with haematological malignancies if not detected and treated early. Clinical symptoms of IPA includes fever, cough, haemoptysis, pleuritic chest pain and dyspnoea. However this clinical symptoms are nonspecific for IPA [5].

IPA diagnosis was categorized in to Possible, Probable and Proven according to European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC-MSG). For diagnosis of Proven Invasive Aspergillosis, it required histopathological confirmation which is often not permissible due to most of the hematological malignancy patient are at risk of bleeding complication for invasive procedure. It also required positive culture which is provide result in a longer time causing delay in diagnosis and actual antifungal treatment to be start [6]. For diagnosis of Probable Invasive Aspergillosis, it required positive clinical criteria and positive mycological test such as positive Galactomannan (GM) assay. No test for histopathological is needed. As consequences, they are recent studies focuses on noninvasive diagnostic tool including Galactomannan and *Aspergillus* – specific polymerase chain reaction (PCR) as mycological marker with medical imaging which is CT thorax findings to support the diagnosis [7-9]. For Possible Invasive Aspergillosis, the diagnosis is made based on clinical history without any positive results in either mycological test or histopathological test positivity.

Galactomannan is a linear hetero-polysaccharide polymer, which make up the specific wall of Fungi phylum. In daily practice, positive blood test for Galactomannan has become one of the mycological criteria accepted in EORTC-MSG. Several studied done regarding Galactomannan showed that GM levels may be lower in patient with

Airway Invasive compared to Angio Invasive and CT thorax as a complementary test for diagnosis of IPA [7, 10].

Aspergillus specific -PCR is a molecular study which is not included in EORTC-MSG criteria yet for diagnosis of Probable IPA because it is not adequately standardized even though it has currently become more accessible. In addition, there is always risk of sample contamination at the patient's bed side or laboratory by airborne spores which is responsible for false positive results. However, the molecular technique seem to arise rapid approach that could be used for diagnosis of IPA. There are two types of the molecular techniques include real time PCR and nested PCR. The development of real-time PCR platforms has enhanced the quality of diagnostic PCR assays. However the preparation is still complex and expensive. Nested PCR is more convenience in preparation but notorious for contamination, but still both of this method was shown to be high degree of correlation to each other[9].

Early CT thorax may provide early sign for Invasive Pulmonary Aspergillosis. It includes Airway Invasive and Angio Invasive type of changes. In Airway Invasive the changes includes tree in bud appearance and consolidation where the organism already infiltrates in to alveoli. As diseases progressed, further lung parenchymal changes are demonstrated including nodule without halo, later once vascular invasion occurred the nodule will have halo sign due to surrounding lung infarction. According to Greene *et al*, the halo sign is visible in 60.9% of Invasive Pulmonary Aspergillosis(IPA) cases, especially in the first week after the onset of infection[11]. In a retrospective study of IPA done by Caillot *et al*., reported that the halo sign was present in 92% of patients[12]. As soon as PMN count is >500/mm^3, the nodules excavate giving an air crescent

sign. This image is usually observed in the immune restoration phase, around the third week of illness evolution. However, no CT thorax image is either sensitive or specific of IPA. For example the halo sign may be observed in other pathological illness such as metastases, bronchoalveolar carcinoma,lymphoma or systemic disease such as Wegener granulomatosis[13].

The Proven IPA only can be diagnosed once patient had histopathological findings which is not permissible to be done in patient with hematological malignancy. Positive *Aspergillus* culture is time consuming causing delayed in diagnosing IPA. Even though the gold standard for IPA diagnosis is Proven IPA, Probable IPA also can be considered as definite diagnosis. With that, the mycological test need to be conjunction with imaging findings for early diagnosis to be made. In advance, the empirical antifungal treatment can be start without any doubt and delay.

The purpose of this study is to determine association of blood serological test for Galactomannan assay and *Aspergillus* specific -PCR with CT thorax findings and further to analyses the predominant CT findings either Airway Invasive or Angio Invasive. Other than that, this study will provide the type of CT thorax findings or changes for patient with Possible and Probable Pulmonary Aspergillosis which be helpful for clinician to diagnosed and start the empirical antifungal treatment in patient who fulfilled clinical criteria for IPA especially in hospital without facilities to provide the mycological test.

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CHAPTER 2: STUDY PROTOCOL

2.1 TITLE

Association of CT thorax findings with Galactomannan and *Aspergillus* - specific Polymerase Chain Reaction for diagnosis of Invasive Aspergillosis in patient with febrile neutropenia.

2.2 OBJECTIVE

2.2.1 General objective

To determine association of CT thorax findings in patient with Invasive Pulmonary Aspergillosis with Galactomannan and *Aspergillus*-specific Polymerase Chain Reaction.

2.2.2 Specific objectives

1. To determine association between CT thorax findings with Galactomannan result in patient with febrile neutropenia.
2. To determine association between CT thorax findings with *Aspergillus*-specific Polymerase Chain Reaction result in patient with febrile neutropenia.

2.3 METHODOLOGY

2.3.1 Study design

This is a cross sectional study conducted at Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian Kelantan from January 2015 until November 2017.

2.3.2 Population and Sample

Reference population: Patient with hematological malignancy.

Source population: Patient with hematological malignancy and febrile neutropenia who received chemotherapy or hematopoietic stem cell transplant.

Study participant: Patient with haematological malignancy who received chemotherapy or haematopoietic stem cell transplant and complicated with persistent febrile neutropenia despite on antibiotic for 3 days or more with CT thorax.

2.3.3 Sampling method

Purposive sampling method.

2.3.4 Inclusion Criteria

1. Patient with haematological malignancy admitted to haematological ward (Age >12 year old).
2. Neutropenia following chemotherapy with absolute neutrophil count (ANC) $<0.5 \times 10^{9/L.}$
3. Persistent fever 3 days or more despite on appropriate broad-spectrum antibiotic given.
4. Patient with CT Thorax within 2 weeks of clinical diagnosis of Possible IPA.

2.3.5 Exclusion Criteria

1. Patients who are positive human immunodeficiency virus (HIV).
2. Patients with positive bacteraemia.

3. Patients with previously diagnosed Proven and Probable invasive fungal infections according to (EORTC/MSG) which is not completed antifungal treatment (8weeks).

4. Patient with concomitant malignancies other than haematological malignancies.

2.3.6 Sample Size Calculation

For both objective 1 and 2:

Sample size is calculated using Sample Size Calculator version 1.7, updated August 2015 from website: www.medic.usm.my/biostat, using 2 proportions-Hypothesis testing.

Proportion in control (p0) = estimated proportion of study outcome in the unexposed group (airway invasive CT finding).

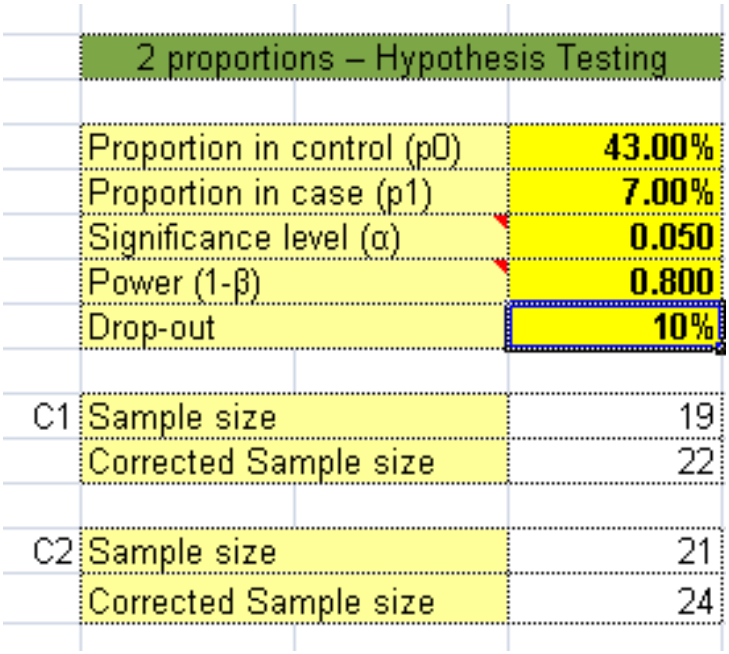
Proportion in case (p1) = estimated proportion of study outcome in the exposed group (Angio Invasive CT finding).

p0 =43% p1 = 7% (M.Weisser, 2005),

Significance level (alpha) 0.05.

Power of study (1- β) of 80%

Total sample size obtained including 10% of drop out is 24 patient.



	2 proportions – Hypothesis Testing	
	Proportion in control (p0)	**43.00%**
	Proportion in case (p1)	**7.00%**
	Significance level (α)	**0.050**
	Power (1-β)	**0.800**
	Drop-out	**10%**
C1	Sample size	19
	Corrected Sample size	22
C2	Sample size	21
	Corrected Sample size	24

Total patient to be sampled taken from sample size calculation is 24.

2.3.7 Research Tools

1. CT scan machine -Siemens SOMATOM Definition AS+ which is capable of producing 128-slices of images per rotation.

2. Picture Archive Communication System (PACS) in HUSM. (PACS Universal Viewer Version 5.0 SP6).

3. Diagnostic workstation with 2 Mega Pixel monitor- (Barco MPG 2121 monitor– resolution 2048 x 1536).

2.3.8 Operational definition

1. Fever: Temperature > 38^0C recorded more than 1 Hour or >38.5^0C recorded once.

2. Neutropenia: Absolute neutrophil count (ANC) < 0.5 x 109/L or< 1.0 x 109/L if rapid decrease was predicted in the following 24-48 hours.

3. Persistent febrile neutropenia: Persistent fever 3 days or more despite of broad-spectrum antibiotic.

4. Possible, Probable and Proven Invasive Aspergillosis: Defined according to the European Organization for Research and Treatment of Cancer-Mycoses Study Group (Figure 1).

-Proven invasive fungal disease (IFD) requires positive mycological criteria.

-Probable IFD requires the presence of a host factor, a clinical criterion, and a mycological criterion.

-Possible IFD requires a host factor and a clinical criterion but for which mycological criteria are absent.

5. CT thorax findings:

Divided in to 2 category:

1: **Airway Invasive:**

- Tree in bud : Peripheral branching structure with ground glass opacity.
- Consolidation: Opacities with obscuration of the underlying vessels.

2: **Angio Invasive.**

- Nodule with halo : Nodule with surrounding ground glass opacities (area of hazy increased attenuation without obscuration of underlying vascular marking).
- Nodule without halo : Nodule with no surrounding ground glass opacities.
- Air crescent sign : Crescent-shaped or circumferential area of radiolucency within a parenchymal consolidation or nodular opacity.
- Cavity : Lucent area surrounded by wall within lung parenchyma.

(Reference: L. Beth Gadkowski, 2008 and Greene *et al*, 2007)

2.3.9 Patients recruitment and mycological test.

- Patient who presented to medical team with haematological malignancy and febrile neutropenia and suspected for Invasive Pulmonary Aspergillosis will be recruited by primary team.
- Blood test for Galactomannan and *Aspergillus* specific -PCR will be perform by medical team.
- Patient are then referred to radiology department by medical team for CT thorax (part of routine investigation) and early appointment date within two weeks will be given .

- Blood sample result for Galactomannan and *Aspergillus* specific -PCR will be trace from microbiology team.

- An optical density (OD) of 0.5 and higher in any serum of Galactomannan sample will be considered positive result (Based on EORTC-MSG criteria).

- Galactomannan result classified into 2 category:

1. Negative: less than 0.5 OD
2. Positive: 0.5 and more OD

2.3.10 Image analysis:

- All subjects underwent CT Thorax using MDCT scanner (SOMATOM Sensation cardiac 128; Siemens AG, Munich, Germany)

-Scan were performed craniocaudally using 69 mAs, 100 - 120 kVp and reconstructed with:

1) B70f (lung setting), Window Width 1200 and Centre -600.
2) B26f (soft tissue setting), Window Width 400 and Centre 40.

- Intravenous iodinated low osmolar contrast media 300 to 320mgI/ml, 70ml and flush with 50ml of normal saline using injector at rate of 3 mls / second.

-Images were reviewed by Radiologist more than 10 years of experienced.

-Blood for Galactomannan and *Aspergillus*-specific PCR result was blinded to avoid bias.

2.3.11 Statistical analysis

Analysis of both objectives (objective 1 and 2) in this study were performed using PASW version 18. We used a Fisher Exact test for categorical variables.

2.3.12 Confidentiality and Privacy

We give serial number for each of the patient. No identifiable data were expressed and shared to the public resulting from this study.

2.3.13 Ethical Consideration

This study was approved by Jawatankuasa Etika Penyelidikan (Manusia) of Universiti Sains Malaysia (JEPeM) which is the Research and Ethical Committee of Universiti Sains Malaysia (JEPeM code: USM/JEPeM/16010006) which complies with the Declaration of Helsinki (Appendix B).

CHAPTER 3: MANUSCRIPT

3.1. TITLE PAGE

ASSOCIATION OF CT THORAX FINDINGS WITH GALACTOMANNAN AND *ASPERGILLUS* - SPECIFIC POLYMERASE CHAIN REACTION FOR DIAGNOSIS OF INVASIVE ASPERGILLOSIS IN PATIENT WITH FEBRILE NEUTROPENIA.

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MAIN DOCUMENT

3.2 TITLE

ASSOCIATION OF CT THORAX FINDINGS WITH GALACTOMANNAN AND *ASPERGILLUS* - SPECIFIC POLYMERASE CHAIN REACTION FOR DIAGNOSIS OF INVASIVE ASPERGILLOSIS IN PATIENT WITH FEBRILE NEUTROPENIA.

3.3 ABSTRACT

Background: The Computed tomography (CT) features of Invasive Pulmonary Aspergillosis (IPA) include Airway and Angio Invasive type. However those features are nonspecific and diagnosis should be made in conjunction with EORTC-MSG criteria. In this study, we evaluated the association between the CT findings and Galactomannan and *Aspergillus* specific PCR in patient with febrile neutropenia.

Methods: Patients with hematological malignancy who had persistent febrile neutropenia of 3 days or more despite of antibiotic underwent CT thorax within 2 weeks of clinical diagnosis of IPA. Blood serum for Galactomannan assay and *Aspergillus* - specific PCR were performed. Changes of CT thorax were documented and association between mycological findings were analyzed using Fisher exact test. Significant association taken at p value of < 0.05.

Results: A total of 26 patients were enrolled in our study. CT findings were positive in 17(65.4%), 8(30.8%) was positive for Galactomannan and 9(34.6%) for PCR. There was

significant association between PCR and CT thorax finding (p value 0.009, 95% CI) but not between Galactomannan and CT Thorax (p value 0.667, 95% CI).

Conclusion: Combination of PCR and CT thorax promised reliable non-invasive tools for early diagnosis of IPA. The *Aspergillus* specific PCR might to be consider as one of the mycological criteria in EORTC-MSG for diagnosis of IPA in the future.

Keywords: *Invasive Pulmonary Aspergillosis, galactomannan, Polymerase chain reaction, Radiography thoracic, febrile neutropenia*

3.4 INTRODUCTION

Pulmonary Aspergillosis is caused by *Aspergillus fumigatus* lung infection. There are four manifestations of *Aspergillus* lung infection which is include Pulmonary Aspergilloma, Allergic Bronchopulmonary Aspergillosis, Chronic Necrotizing or Semi Invasive Aspergillosis and Invasive Aspergillosis[1]. The manifestations of *Aspergillus* lung infection depends on an individual immune response.

The most important spectrum in *Aspergillus* infection is Invasive Pulmonary Aspergillosis (IPA) where it affects severely immunocompromised hosts. It first described by Rankin and was published in1953. In early course of disease, it will affect the airway (Airway Invasive Aspergillosis) before further vascular invasion (Angio Invasive Aspergillosis) causing necrosis of the lung parenchyma.

The diagnosis of Invasive Pulmonary Aspergillosis (IPA), continues as a challenging diagnosis to some groups of patient especially whom with underlying haematological malignancies. The worst is, most of the hematological malignancies patients with febrile neutropenia are at greatest risk for fungal infection. The common organism is *Aspergillus* and its incidence was estimated around 10-15%[2]. The delayed in diagnosis and initiation of empirical antifungal treatment will lead to increasing in severity of IPA and in turns associated with high morbidity and mortality [3].

Based on European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC-MSG) diagnosis of IPA was categorized into three which is Proven IPA, Probable IPA and Possible IPA. Histopathological or cultured based of *Aspergillus* is the gold standard for diagnosis of Proven IPA, whereas the diagnosis of

Probable IPA is made when Galactomannan (GM) is positive. The diagnosis of Possible IPA only required clinical evidence of IPA as included in EORTC-MSG criteria.

Galactomannan is a linear hetero-polysaccharide polymer, which make up the specific wall of Fungi phylum. In daily practice, positive blood test for Galactomannan has become one of the mycological criteria accepted in EORTC-MSG. This is because GM has high specificity (85 – 99%) even though the sensitivity is broad (29 – 94%) [2, 4].

Aspergillus specific -PCR is a molecular study which is not included in EORTC-MSG criteria yet for diagnosis of Probable IPA because it is not adequately standardized even though it has currently become more accessible. In addition, there is always risk of sample contamination at the patient's bed side or laboratory by airborne spores which is responsible for false positive results. However, the molecular technique seem to arise rapid approach that could be used for diagnosis of IPA. There are two types of the molecular techniques include real time PCR and nested PCR. The development of real-time PCR platforms has enhanced the quality of diagnostic PCR assays. However the preparation is still complex and expensive. Nested PCR is more convenience in preparation but notorious for contamination, but still both of this method was shown to be high degree of correlation to each other[5].

There were few recent studies that compare the GM and PCR performance in patient with IPA, showed that PCR had high sensitivity and specificity up to 100% and 88% respectively[6, 7]. However both of that study was not correlate with CT Thorax findings.