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Comparison between Polymerase Chain Reaction, Haematoxylin and Eosin and Quick-Diff Stains in the Diagnosis of *Madurella Mycetomatis* in Sinnar State-Sudan (2014-2015)

Nuha Y. Ali [1], Rania M. SidAhmed [2], Nagwa A. Mahmoud [3]
[1]*Department of Histopathology and Cytology, Faculty of Medical Laboratory Sciences, University of Gezira - P.O.Box 20 - Wad Medani,Sudan.*
[2]*Department of Histopathology and Cytology, Faculty of Medical Laboratory Sciences, University of Gezira - P.O.Box 20 - Wad Medani,Sudan.*
[3]*Department of Microbiology, Faculty of Medical Laboratory Sciences, University of Khartoum, Sudan*.

Abstract: Madurella Mycetomatis is a chronic, specific, granulomatous, progressive inflammatory disease; it usually involves the subcutaneous tissue after a traumatic inoculation of the causative organism. Mycetoma may be caused by true fungi or by higher bacteria. In Sudan; Madura is spread mainly in agricultural and grazing areas like Gezira and Sinnar states. This study aimed to compare between Polymerase Chain Reaction (PCR), Haematoxylin and Eosin (H&E), and Quick-Diff Stains in the diagnosis of Madurella Mycetomatis. Samples of fine needle aspiration cytology were collected from infected site from patients that were clinically diagnosed with Madurella Mycetomatis in Sinnar hospital. These samples were diagnosed using H&E and Quick-Diff stains of cytology and PCR techniques. The results showed that the positivity of the infection using H&E and Quick-Diff stains for cytology was only 26% and the remaining 74% gave negative results for each stain, whereas the positivity of the infection using PCR technique was 86% and only 14% of the total cases gave negative results. According to the Receiver Operating Curve, which was involved in assessing the specificity and sensitivity of the three methods; there was a difference between the three methods. H&E and Quick-Diff stains for cytology showed 32.1% sensitivity and 100% specificity, Whereas PCR technique showed 93.3% sensitivity and 100% specificity. In conclusion PCR technique was superior to H&E stain and Quick-Diff stains for the diagnosis of Madurella Mycetomatis. Local health care facilities and health education must be sufficient and adequate in areas where Madurella Mycetomatis is endemic, because the morbidity is massive and enormous, and it has many clinical and socio-economic impacts on patients, families, and the community.
Key words: *Madurella Mycetomatis Diagnosis; H&E; Quick-DIFF; PCR; Sudan*

I. Introduction

The morbidity caused by *Madurella Mycetomatis (M. Mycetomatis* or Madura*)* is massive and enormous. It has many clinical and socio-economic impacts on patients, families and the community. In areas where *Madurella Mycetomatis* is endemic, local health care facilities and health education are usually insufficient and inadequate. Also it is a common medical and health problem in Sinnar state, therefore more sensitive and specific diagnostic tools are needed for accurate detection of *Madurella Mycetomatis*. The aims of this study to compare between Polymerase Chain Reaction (PCR), Heamatoxylin and Eosin (H&E), and Quick-Diff stains (RAL) in the diagnosis of *Madurella Mycetomatis*, to identify the accurate method to be used in the diagnosis of Mycetoma, and to assess the sensitivity and specificity of the three methods [(1)].

Madurella Mycetomatis is usually classified into eumycetoma and actinomycetoma respectively [(1)]. Tumefaction and formation of sinus tracts characterized of *Madurella Mycetomatis*. The sinuses usually discharge purulent and seropurulent exudate containing grains. It may spread to involve the skin and the deep structures resulting in destruction, deformity and loss of function very occasionally it could be fatal [(2)].

The true incidence and the geographical distribution of *Madurella Mycetomatis* throughout the world is not exactly known due to the nature of the disease which is usually painless, slowly progressive, which may lead to the late presentation of the majority of patients. It is endemic in tropical and subtropical regions. The African continent seems to have highest prevalence [(3)]. Male predominance is a constant finding in *Madurella Mycetomatis* with a sex ratio of 3.7: 1. This is commonly attributed to the greater risk of exposure to organisms in the soil during the outdoor activities. No age is exempted but commonly affects adults between 20-40 years of age and these are the earning members of the society especially in under developed countries. However, in endemic regions children and elderly people may also be affected [(4)]. *Madurella Mycetomatis* is seen more conventionally in cultivators, field laborers and in herdsmen who come in contact with the land in endemic areas people of other occupations are affected [(5) (6)].

In Sudan Madura is widely spread mainly in agricultural and grazing areas like Gezira and Sinnar states with adults being the mostly affected because they work on fields so they are most susceptible to infection by the causative agents as a result of that , it is one of the most economically dangerous diseases , moreover , the treatment is expensive also, it often and by amputation of the affected organ so all these are seriousness factors of the infection, and what ensures the seriousness of the infection is that the number of patients is high however it decrease every years for instance , according to Soba Center of Mycetoma, which is a national center, the number of cases in 2013 is 4800 decreases in very good and remarkable way to approximately 1500 in 2014 , 60 % of them is between 15-35 years old under poverty line, but that doesn't mean it not a serious number because this is only the registered number of cases who came to the center. In Gezira state alone the number of patients is about 7480 from thousands of patients considered to be in 2013 that make Gezira state nearly most affected area in world followed by Sinnar state [(7)].

In Sudan fine needle aspiration (FNA) of *M. Mycetomatis* was described for the first time by El hag, Fahal and Gasim in a paper published in (Acta Cytologica 1996; 40:461-464). The smears stained by H&E stain. Lesion has a distinct appearance in a cytology smear characterised by the presence of polymorphous inflammatory cells. The small-subunit ribosomal rRNA sequences utilized for many investigators for the determination of species of fungi and bacteria [(8)].

II. Definition Of Study And Study Area

This was prospective and cross-sectional study in diagnosis of *M. Mycetomatis* using Haematoxylin and Eosin (H&E) stain, Quick-Diff stains (RALL 555) and molecular technique (PCR). The study was conducted during the period from October 2014 to August 2015.

Study samples were selected from Sinnar State, which is a part of the Blue Nile region in South-East Sudan and is delimited by Gezira State in the North, The Blue Nile in the South, Algadarif State and the Sudanese Ethiopian borders in the East and the White Nile State in the West. The area approximately is 40,680 kilometers square with population 1,400,000 persons. The state contains seven localities and Singa city is the capital of the state (sudan.gov.sd, 2012). Volunteer patients with *Madurella Mycetomatis* were participated in this study. These fine needle aspirations of samples were brought from all over the catchment area for different techniques.

III. The Ordinary Haematoxylin And Eosin (H&E) Stain

The smears are hydrated in descending grades of alcohol concentration, at 95% through 70% to distilled water for 2 minutes in each stage. For staining of the nucleus, the sections treated with Mayer's Haematoxylin for 8 minutes and differentiated by rinsing in acid alcohol for seconds, bluing in running tap water for 8minutes, counterstaining in Eosin for 1 minute, and rinsed in water. The sections dehydrated in 70% alcohol through 95% and 100% alcohol, and then blotted in a filter paper, cleared in xylene and mounted in DPX, after that the smears were ready for microscopic examination.

Interpretation of the results:
Presence of *Madurella Mycetomatis* and polymorphous inflammatory cells by H&E stain [(9)].

IV. The Diff Quick Stain (RALL 555)

The smears were air dried, rinsed in methanol for 5 times, then rinsed in Eosin 5 times and also rinsed in Giemsa stain for 5 times, and finally allowed to dry.
Interpretation of the results:
Presence of *Madurella Mycetomatis* and polymorphous inflammatory cells by Diff Quick stain [(9)].

V. Molecular Method

5.1 DNA Extraction

DNA will be extract from 50 different samples about 10 mg fugal were crushed in mortor and add 490 ml CTAB buffer (2% CTAB , 100 MmtRIS HCL , 20 mM EDTA , 1,4 M Nacl)
About 10 µll of proteinase K were added and the mixure were mixed in MoBio vortex some minutes.
The tubes were incubated at 60 C for 60 minutes and re-mixed again with MoBio vortex to insure mycelia distruption.
Five hundred microliter of chloroform : isoamylalcohol (24:1) were added followed by shaking for two minutattes +50 µl Na acetate.
Tubes were spin in 14.000 rpm microfuge for 10 minutes and the upper layer was collected in new sterile tube with 0.55 ice cold iso-propanol shaking and respine.
Finally, the pellets were washed with 70 % ethanol, left for air dry and re-suspended in 100µl TE buffer [(10)].

5.2 PCR Procedure

Prepare a PCR mixture (20 µl) containing 2 µl DNA,1 µl primer (f:10 pmol\20 µl 20 pmol primer 26.1A(5,AATGAGTTGGGTTTAACGG-3.) and 21 pmol primer .28-3A (5-TCCCGGTAGTGTAGTGTCCCT- 3~) 16 µl DW.

Perform the PCR amplification in thermocycler using a cycle program with 40 cycles of 94 c for 2 min denaturation 94^{o}C for 20 sec Annealing 65^{o}C Extension 72^{o}C 20 sec Final Extension 72^{o}C 30 sec.

Separate the PCR products on 1% agarose gel in electrophoreses machine and reading of the results [10].

VI. Results

6.1 Diagnosis Ofmycetoma Using H&E And Diff-Quick Stains

From 50 patients whom diagnosed clinically with *Madurella Mycetomatis*; there are 13 (26%) cases give positive results and 37 (74%) cases give negative results using H&E and Diff- Quick stains (figure 6.1).

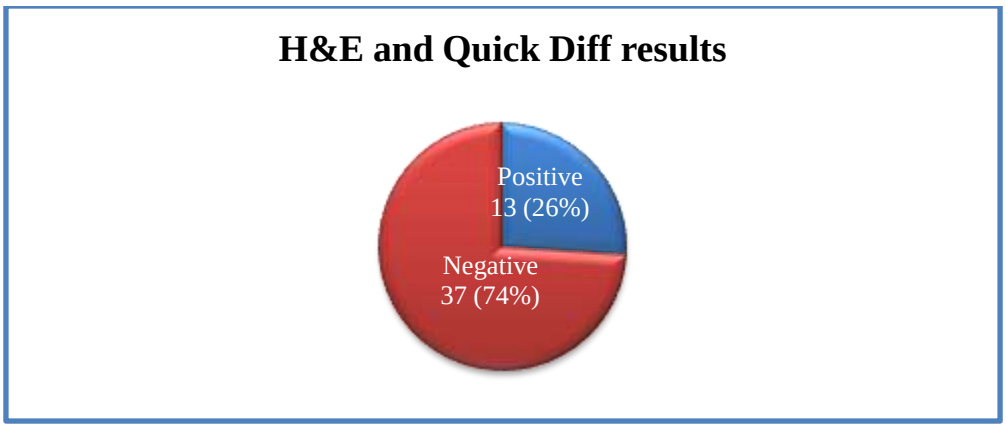

Figure (6.1): Diagnosis of *Madurella Mycetomatis* using H&E and Quick Diff stains.

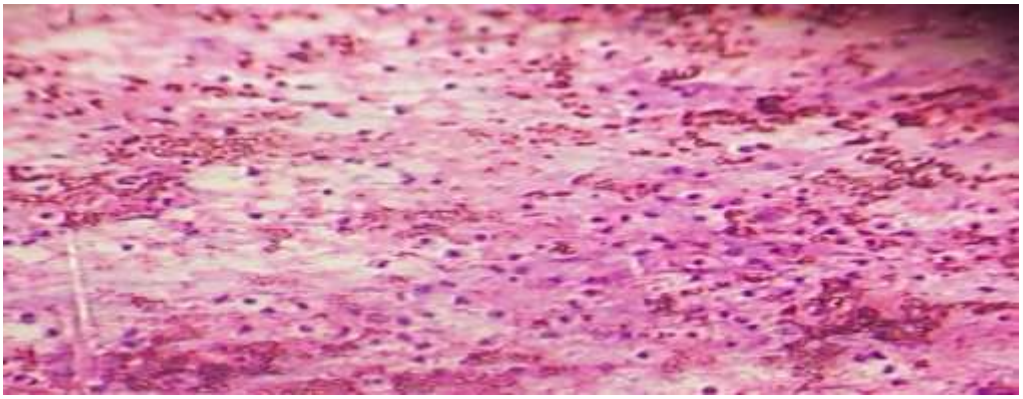

Figure (6.2): *Madurella Mycetomatis* using H&E stain

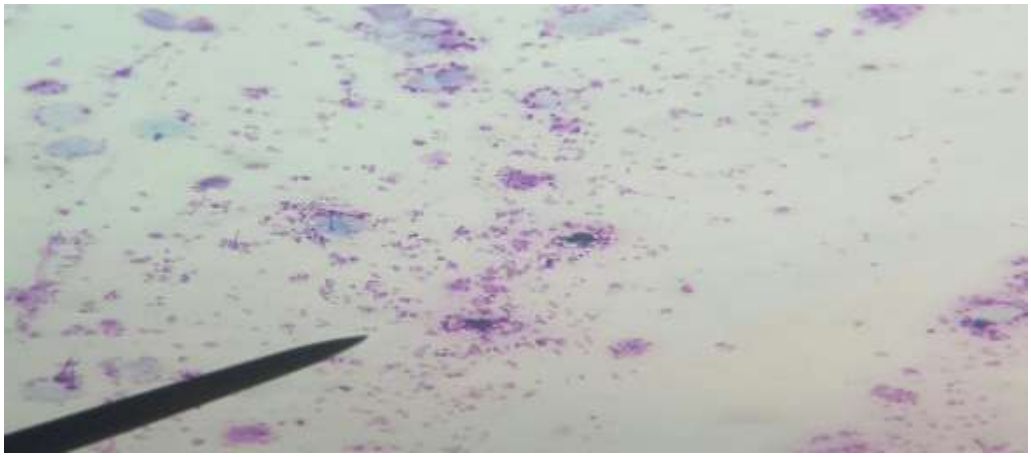

Figure (6.3): *Madurella Mycetomatis* using Quick Diff stains

6.2 Diagnosis Of Mycetoma Using PCR

Out of 50 patients whom diagnosed clinically with *Madurella Mycetomatis*; there are 43 (86%) cases give positive results and 7 (14%) cases give a negative results using a PCR technique (figure 6.4).

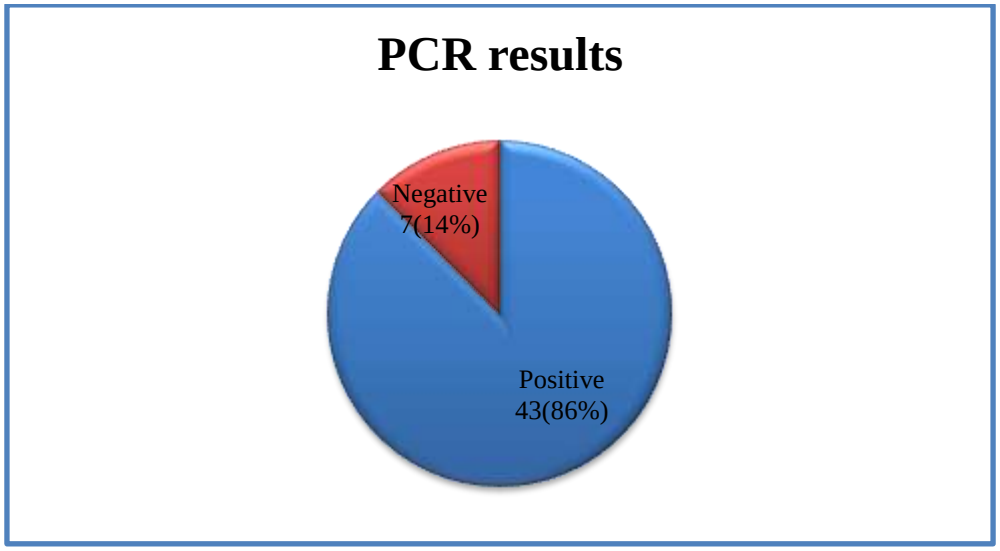

Figure (6.4): Diagnosis of *Madurella Mycetomatis* using PCR technique

Figure (6.5): The result *Madurella Mycetomatis* using PCR technique

6.3 THE SENSITIVITY AND SPECIFICITY OF H&E, QUICK-DIFF, AND PCR

Receiver Operating Characteristics (ROC) curve to H&E stain was used to measure specificity and sensitivity of the test compared to a gold standard method.

The curve shows that specificity of H&E stain was 100% and the sensitivity was 32.1%, while the specificity of Quick Diff stains was 100% but the sensitivity was 32.1%. The sensitivity of PCR is 93.3% and the specificity is 100% and Area under the ROC curve (AUC) is 0.97, whereas the sensitivity and specificity of cytological technique using H&E and Quick Diff stains are 32.1%, 100% respectively and Area under the ROC curve (AUC) is 0.66 (figure 6.6, 6.7, 6.8).

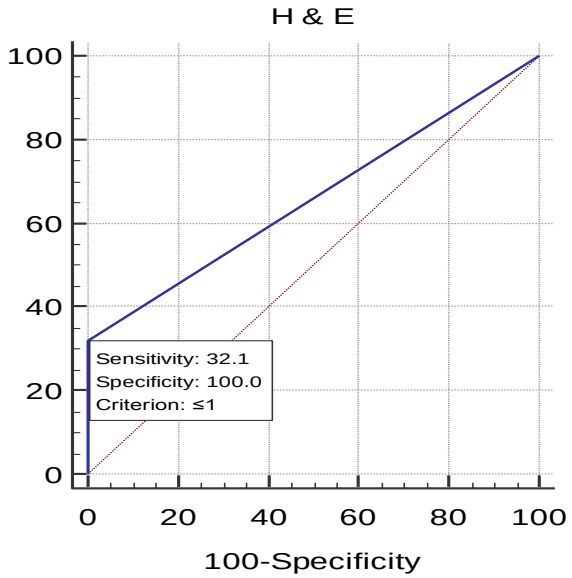

Figure (6.6) Specificity and sensitivity of cytological test using H&E stain.

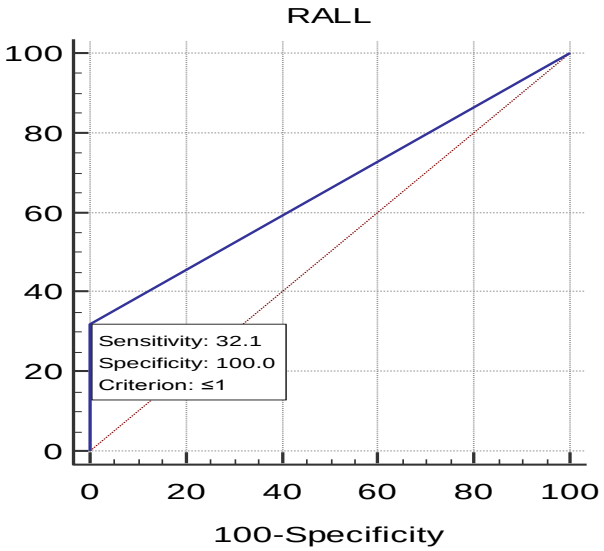

Figure (6.7) Specificity and sensitivity of cytological test using Quick Diff stains.

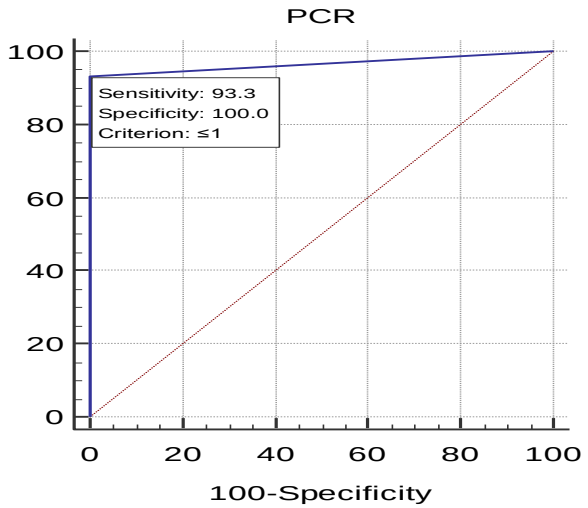

Figure (6.8): Specificity and sensitivity of PCR.

VII. Dissicusion

Fifty samples of fine needle aspiration cytology were collected randomly from infected site clinically diagnosed with *Madurella Mycetomatis* in Sinnar state. These samples were diagnosed using Haematoxylin and Eosin (H&E), Quick Diff stains of cytology and polymerase chain reaction (PCR) techniques. The results showed that polymerase chain reaction (PCR) technique was more accurate. The positivity of the infection using PCR techniques was 86% and only 14% of cases gave negative results. Whereas the positivity of the infection using Haematoxylin & Eosin and Diff-Quick stains of cytology was only 26% and the remaining 74% gave negative results. According to the Receiver Operating Curve, which is involved in assessing the specificity and sensitivity of the three methods; there was a difference between the methods. PCR technique showed 93.3% sensitivity and 100% specificity. Whereas H&E and Quick-Diff stains of cytology showed 32.1% sensitivity and 100% specificity.

H&E stain and Quick Diff stains were used for cytopathological diagnosis, which deal with morphology of the grains and inflammatory reaction of the tissues. Their staining mechanism of H&E and Quick-Diff stains depends on acid –base reaction between the stain and the tissues. Molecular technique is a technique for localizing of specific gene in the specimen using specific primer for the target gene. PCR was more sensitive than H&E and Quick Diff, and this was well reflected in our results.

These results agree with many authors like Sanjay Mukhopadhyay ***e****t al.* (2010), compared between H&E and PCR in the diagnosis of mycetoma. H&E is used for morphological appearance, but unfortunately, the morphologic appearance of mycobacteria on histologic sections is not reliable for species. Pathologists and clinicians are sometimes faced with the difficult situation whereby mycobacterium are identified by histological methods but speciation is not possible because no specimen was submitted for culture or culture results are negative, therefore the role of PCR and other molecular methods for detection and speciation of Mycobacteria, which has importance in the treatment [(11)]**.**

Vera-Cabrera L, *et al.* (2012), said that the diagnostic tools have been developed to aid in the diagnosis of Mycetoma, but most of them cannot be used alone. Clinical examination and different imaging techniques can determine the extent of the disease and, to some extent, its type. A first indication of the causative agent can be obtained by isolating grains from lesions with a fine needle aspirate. Further assessment can be achieved by simply looking at the color of grains. More reliable data can be gleaned by looking at grains histologically, but the definite identification of causative agents of mycetoma should be done only after culture and preferably by using appropriate molecular tools [(12)].

Shagufta Tahir Mufti and Hessa Aljhdali (2015), they studied the histopathological and clinical correlation of mycetoma among patients attending King Abdulaziz University Hospital. The data of all histopathologically diagnosed mycetomas in the period between January 1998 and January 2013 were collected through a computerized database search of the anatomic pathology archives at King Abdulaziz University Hospital. The collected data were analysed. Identification of species was performed for five patients using 16S ribosomal DNA and internal transcribed spacer 2. They concluded that actinomycetoma is more common than eumycetoma in this region. The disease has to be prioritized again and more robust and quick molecular diagnostic tools should be made available in order to save patients form disfiguring amputations [(13)].

Wehrhahn, *et al.* (2012), published that difficulties associated with the accurate identification of the known causative agents of actinomycetoma using phenotypic criteria promoted the development and application of molecular diagnostic procedures. Reliable identification of causal agents to the genera *Actinomadura*, *Nocardia*, and *Streptomyces* can be achieved by PCR and sequencing of conserved genes, notably, by comparison of almost complete 16S rRNA gene sequences of isolates against corresponding sequences of their phylogenetic neighbours drawn [(14)].

Yousif BM, *et al.* (2010), published that grains need to be isolated from lesions in order to identify causative agents of mycetoma to the species level. This is usually performed by a deep-seated biopsy or with fine needle aspiration cytology (FNAC) was described in 1991 and fully evaluated by Yousif and colleagues in 2010. In this technique the material is usually bloody and is therefore left to clot, then examined either by eye or microscopically after fixation in 10% formalin-saline and staining with haematoxylin and eosin (H&E). 230 mycetoma patients were examined using FNAC and the cell block technique to examine the grains of *Actinomadura madurae*, *Actinomadura pelletierii*, *Streptomyces somaliensis*, and *Madurella mycetomatis* and the tissue response to mycetoma. The technique had sensitivities of 87.5% and 85.7% for eumycetoma and actinomycetoma, respectively [(15)].

VIII. Conclusion

PCR technique was superior to H&E and Quick Diff stains in the diagnosis of *Madurella mycetomatis*, also it was more sensitive than H&E and Quick Diff stains.

Due to its high accuracy, PCR recommended to confirm the diagnosis of Mycetoma.

Local health care facilities and health education must be sufficient and adequate in areas where mycetoma is endemic, because the morbidity is massive and enormous. And it has many clinical and socio-economic impacts on patients, families and the community.

Further studies including a larger population size should be done.

Ethical Approval

Ethical approval for this study was obtained from the research ethical committee from the Gezira State Ministry of Health and the hospital administration. Each participant was asked to sign a written ethical consent form during the interview, before the specimen was taken. Consents were taken from patients to take samples after agreement of the participant, numbered samples, and no patients' name.

Competing Interests

Authors have declared that no competing interests exist.

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