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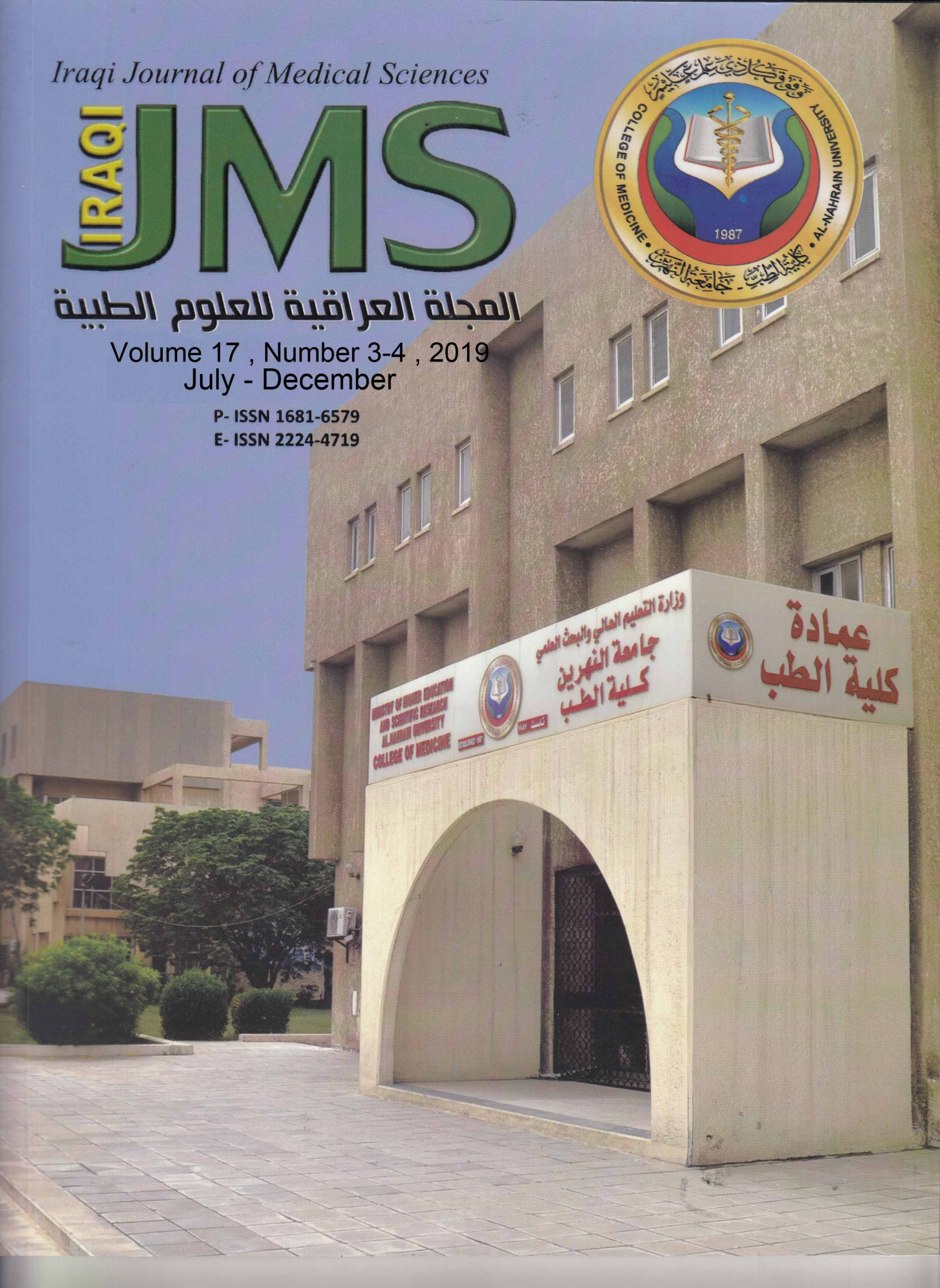

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Issued Quarterly

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Asymmetric Dimethyl Arginine and Uromodulin in the Chronic Kidney Disease

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Dept. of Chemistry and Biochemistry, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Asymmetric dimethyl arginine, symmetric dimethyl arginine and uromodulin used as early biomarkers of diagnosis renal diseases. The early stages side effects of inveterate kidney malady are ordinarily not clear. Noteworthy decrease of the kidney work is the primary self-evident sign of infection. On the off chance that analyzed early stages 1 to 3, the movement of unremitting kidney infection can be changed and complications diminished. In stages 4 and 5 broad kidney harm is watched, which as a rule comes about in end-stage renal disappointment.

Keywords Symmetric Dimethyl arginine, uromodulin and kidney

Citation Ali NM. Asymmetric dimethyl arginine and uromodulin in the chronic kidney disease. Iraqi JMS. 2019; 17(3&4): 166-167. doi: 10.22578/IJMS.17.3&4.1

Asymmetric Dimethyl arginine (ADMA)

Asymmetric dimethyl arginine (ADMA) may be a modern biomolecule that can conceivably utilize as a biomarker in incessant kidney illness (chronic kidney disease). It is a simple chain of L-arginine which normally happens in human circulation. It has been appeared that expanded levels of ADMA restrain nitric oxide union and thus it disables endothelial work invigorating renal disability [1]. Agreeing to considers, ADMA levels anticipated a more quickened course of renal work misfortune and advanced the improvement of renal harm due to the reality that it activated glomerular hypertension, endothelial harm, salt amassing, and cell senescence [1,2]. There are a few conceivable atomic instruments of ADMA association in renal impedance. Koyner et al. [3] have recommended that hoisted plasma concentration of ADMA is related with levels of NG-dimethyl arginine dimethyl amino hydrolase (DDAH) protein which metabolizes ADMA and expanded quality expression of chemical protein methyl transferase (PRMT) which produces advertisement.

Symmetric Dimethyl arginine (SDMA)

Symmetric dimethyl arginine (SDMA) may be a steady catabolic item of post-translationally methylated arginine-containing proteins which plays a crucial part in fundamental cellular digestion system. SDMA is killed basically by the kidneys [4]. Higher concentrations of both SDMA and ADMA in hemodialysis patients. Serum and pee concentrations of SDMA have been appeared to relate with kidney brokenness evaluated on the premise of glomerular filtration rate (GFR) and creatinine clearance [5]. Kidney work weakening was related in that consider with the increment in SDMA levels. Too, an expansive meta-analysis of 18 thinks about detailed profoundly critical relationship between SDMA and kidney work. Concurring to ponders, non-renal components counting muscle mass, count calories, irritation, diabetes, and estrogen treatment

had no critical effect on SDMA concentration [4].

Uromodulin

Uromodulin may be a glycoprotein, which according to ponders is likely locked in within the assurance of tubular cells from climbing urinary tract infections included in incessant pyelonephritis and urolithiasis. It is created within the tubular cells of the thick rising appendage and the early distal tubule and discharged into the tubular lumen where it forms a layer on the tubular cell surface. Uromodulin is profoundly copious in pee. It is additionally discharged in tubular cells into the interstitium, be that as it may, its physiological part there remains unknown [5]. Diminished urinary and serum concentrations of uromodulin are found in people with interstitial fibrosis or tubular decay within the course of inveterate kidney malady. The most elevated concentrations of uromodulin in people without CKD were recommended to be due to the reality that no avoidance component for tubular work exists in opposite to glomerular filtration [4]. It has been proposed that plasma uromodulin seem serve as a marker for kidney work in both.

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Higher ST-Segment Elevation in Lead III Than Lead II in Acute Inferior Myocardial Infarction Can Be A Predictor of Short-Term Morbidity and Mortality

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Abstract

Background The incidence of mortality and complications are high in patients with acute inferior wall ST-segment elevation myocardial infarction with right ventricular involvement, which has been reported to be an independent predictor of significant complications and in-hospital mortality.

Objective To investigate the feasibility of using electrocardiographic changes in inferior myocardial infarction represented by ST-segment elevation ratio in lead II and III as a predictor of right ventricular infarction and in-hospital morbidity and mortality.

Methods Ninety-nine patients were studied in this prospective study, their ages ranged from 19-90 years, average 58.12 (±12.7 SD). They were presented to the Coronary Care Unit of Basrah General Hospital with acute inferior ST-segment elevation myocardial infarction. The 12 leads plus right-sided precordial electrocardiograms were done to all patients within 12 hours of the onset of symptoms, and ST-segment elevation was measured. ST-segment elevation in lead III exceeding lead II was defined as a ratio of elevation in lead III: II>1. Patients grouped according to ST-segment elevation III:II ratio into either >1 or ≤1. In-hospital morbidity and mortality were studied in both groups.

Results ST-segment elevation ratio > 1was detected in 68 patients (68.7%) with acute inferior myocardial infarction at time of admission. Right ventricular infarction was diagnosed in 33 (33.3%) patients, with the majority (32 patients) have ST-elevation ratio > 1. Thirty-Six patients had at least one of the in-hospital complications with significantly higher incidence (51.4%) in patients with higher ST elevation ratio. The mortality was statistically higher when ST segment elevation level in the lead III > than that in the lead II.

Conclusion In patients with inferior STEMI, ST-segment elevation in the lead III more than lead II can be a potential marker of the presence of right ventricular infarction in association with inferior myocardial infarction. Short-term prognosis is possibly worse in the presence of a higher ratio between lead III and II ST-segment elevation. However, further studies are needed to validate this conclusion.

Keywords Inferior MI, right ventricular infarction, ST-segment changes

Citation Al-Mansouri LA, Al-Obaidi FR, Al-Humrani AH. Higher ST-Segment elevation in lead III than lead II in acute inferior myocardial infarction can be a predictor of short-term morbidity and mortality. Iraqi JMS. 2019; 17(3&4): 168-174. doi: 10.22578/IJMS.17.3&4.2

List of abbreviations: ECG = Electrocardiogram, LAD = Left anterior descending coronary artery, LCX = Left circumflex coronary artery, LV = Left ventricle, MI = Myocardial infarction, RCA = Right coronary artery, RV = Right ventricle, PCI = Percutaneous coronary intervention, DM = Diabetes mellitus, RVMI = Right ventricle myocardial infarction

Introduction

Acute inferior wall ST-segment elevation myocardial infarction (STEMI) is associated with right ventricular

involvement in 30% of cases [1]. Right ventricular (RV) infarction is an independent predictor of complications with an increased risk of death, shock, ventricular tachycardia, ventricular fibrillation, and atrioventricular block. In 80% of acute inferior wall myocardial infarction (MI) cases, the infarct-related artery is right coronary artery (RCA), which is associated with a higher risk of complications, while it is left circumflex coronary artery (LCX) in the rest. Determination of the infarct-related artery in acute MI is essential to predict potential complications.

Furthermore, predicting the probable site of occlusion within RCA is worthwhile because proximal occlusions are more likely to cause greater myocardial damage [2]. Easily recognizable electrocardiogram (ECG) findings that identify high-risk culprit lesion may facilitate the initial management of patients with inferior wall acute MI. Using ECG can provide timely identification of the infarct-related artery and even the location of the culprit lesion within the infarct-related artery [3]. Hospitals without catheterization laboratory need more available tools such as detailed ECG analysis to define high-risk patients with a large jeopardized myocardium [4]. There are many ECG patterns to indicate extensions of the infarction that are associated with different clinical outcomes and necessitate various therapeutic approaches to be applied [5]. The standard 12-lead ECG does not define the RV territory well. Several different additional lead applications may be used to determine RV injury, including a complete reversal of the standard left-sided precordial leads (resulting in V1R through V6R) or the simplified approach using only V4R. In either case, the degree of ST-segment elevation in the right-sided leads may be of a small magnitude because of the relatively smaller RV muscle mass [6]. Therefore, ECG criteria have been suggested to define the infarct-related artery including ST-elevation ratio between leads II and III. Higher elevation of ST segment in lead III in comparison with lead II (ST elevation III > ST elevation II) is a possible indicator of the RCA as the culprit artery while ST-segment elevation in the lead III less than in lead II (ST elevation III < ST elevation II) may indicate the LCX as the probable culprit artery [7,8].

The objective of this study was to investigate the feasibility of using electrocardiographic changes in inferior myocardial infarction represented by ST-segment elevation ratio in lead II and III as a predictor of right ventricular infarction, in-hospital morbidity and mortality.

Methods

Ninety-nine patients were included in this prospective study; their ages ranged from 19-90 years, mean age 58.12 years (±12.7 SD), presented to the Coronary Care Unit of Basra General Hospital with Acute Inferior STEMI from March 2009 to April 2010. The 12 leads plus right-sided precordial ECG was recorded within 12 hours of presentation, and ST-segment elevation was measured. Myocardial infarction was defined according to WHO criteria [9]. Inferior STEMI was determined by ST-segment elevation >1 mm in 2 or more of leads II, III, and aVF on the baseline ECG. Right Ventricular infarction diagnosed with ST elevation >1 mm in the V4R lead. ST-segment elevation in lead III exceeding lead II was defined as a ratio >1. Patients grouped according to ST-segment elevation III: II ratio into either >1 (Group 1) or ≤1 (Group 2). In-hospital morbidity and mortality; including the incidence of death, cardiogenic shock (blood pressure of ≤ 90/60 mmHg with evidence of decreased organ perfusion) [10] and arrhythmias; were studied in both groups. For each of the 99 patients, the clinical characteristics (including the history of hypertension, diabetes mellitus (DM), ischemic heart diseases, and smoking) and demographics were analyzed. Investigations were done to all patients including serum Troponin, and blood sugar. Management of patients with STEMI mainly pharmacological with thrombolytic therapy as invasive therapy (primary percutaneous coronary intervention (PCI)) is not available at our hospital. Other medications were received by the patients include aspirin, clopidogrel, lipid-lowering agents, IV infusion of

unfractionated heparin, IV normal saline in patients with hypotension and treatment of hypertension and DM.

Results

ST-segment elevation ratio was >1 in 68 patients (68.7%) with acute inferior STEMI at time of cardiac care unit admission and ≤1 in 31 patients (31.3%). A higher number of patients (n=41, 41.4%) with ST-segment elevation ratio >1 were older (more than 60 years) with more male patients in this group than female (Tables 1 and 2).

Table 1. Distribution of the patients according to age

Age (Years)	Group1 No. (%)	Group2 No. (%)	Total No. (%)	*P* value
<40	3 (50%)	3 (50%)	6 (100%)	0.69
40-49	13 (68%)	6 (32%)	19 (100%)	0.159
50-59	11 (52%)	10 (48%)	21 (100%)	0.86
>60	41 (77%)	12 (23%)	53 (100)	0.001

Table 2. Distribution of the patients according to sex

Sex	Group1 No. (%)	Group2 No. (%)	Total No. (%)	*P* value
Male	51 (68%)	24 (32%)	75 (100%)	0.0001
Female	17 (70%)	7 (30%)	24 (100%)	0.084

The prevalence of diabetes was significantly higher in group 1 than group 2, (30.3%) vs. (10.1%) respectively. The prevalence of hypertension (29.2% vs. 9%), history of ischemic heart disease (20.2% for vs. 9%) and smoking (29.2% vs. 12.1%) were significantly higher in group 1 than group 2, respectively (Table 3).

Table 3. Risk factors of the patients

Findings	Group1 No. (%)	Group2 No. (%)	*P value*
Diabetes Mellitus	30 (30.3%)	10 (10.1%)	0.001
Hypertension	29 (29.2%)	9 (9%)	0.001
Past Hx of IHD	20 (20.2%)	2 (2%)	0.004
Smoking	29 (29.2%)	12 (12.1%)	0.001

Right ventricular involvement rate was 33 (33.3%) overall, with 32 patients (32.3%) in group 1 and only one patient (1%) in group 2; there was a highly significant association between Right ventricle myocardial infarction (RVMI) and ST elevation ratio >1, p=0.0001 (Table 4).

Table 4. Comparison of Right ventricle myocardial infarction risk according to ST-segment elevation ratio

Findings	Group1 No. (%)	Group2 No. (%)	Total No. (%)
Positive	32 (96%)	1 (4%)	33 (100%)
Negative	36 (54.5%)	30 (45.5%)	66 (100%)
Total	68 (68.6%)	31 (31.4%)	99 (100%)

p=0.0001

Thirty-six patients (36.3%) had at least one of the in-hospital complications, and a significant association was identified with group 1 as 35 patients (35.3%) were in group1 vs. one patient (1%) in group 2, p=0.0001 (Table 5).

Table 5. Comparison of in-hospital complications risk according to ST-segment elevation ratio

Findings	Group1 No. (%)	Group2 No. (%)	Total No. (%)
Present	35 (97.2%)	1 (2.8%)	36 (100%)
Absent	33 (52.3%)	30 (47.7%)	63 (100%)
Total	68 (68.6%)	31 (31.4%)	99 (100%)

Discussion

This study aims to determine the utility of ECG criteria suggested by previous studies to predict prognosis in patients with acute inferior MI [6, 8]. The rate of inferior MI is about 40–50% of all infarctions, with short-term mortality rates, ranging from 2-9% [11]. The overall survival of inferior STEMI is better than anterior STEMI, but when inferior STEMI is complicated by RVMI; particularly in those with ventricular arrhythmias [12]; the mortality is increased [13]; other significant predictors of six months mortality included age, female gender, diabetes, angina, and stroke [3]. The standard 12-lead ECG plus right-sided leads is a useful screening tool for RVMI complicating inferior STEMI, which has prognostic implications as an independent predictor of poor outcomes compared to anterior STEMI and inferior STEMI without RVMI [14]. The incidence of RVMI in acute Inferior STEMI in this study was 33.3% which was consistent with other studies [15,16]. The association between the incidence of RVMI and ST-segment elevation ratio more than 1 was statistically significant. ST-segment elevation in the lead III more than lead II might suggest the involvement of the right coronary artery rather than the left circumflex artery [17]. Calculation of this ratio may be a useful screening tool for RVMI with the likelihood of RV MI with inferior MI is low in patients with ST-segment elevation in lead III<II [2].

This study showed a 19.1% in-hospital mortality in group 1 as compared with 0.0% in group 2 patients. ST-segment elevation in lead III>II have associated with a statistically significant (p=0.008) higher in-hospital mortality, which is consistent with previous studies [18,19]. The possible explanation for higher mortality rate is increased incidence of ventricular tachycardia and ventricular fibrillation, as the right ventricle may be more arrhythmogenic than the left ventricle in acute ischemia [20]. The overall incidence rate of in-hospital complications

(cardiogenic shock, high degree heart block, VT, VF, AF) is 36.3%. In-hospital complications were significantly higher in group 1 as compared with group 2, indicating the potential value of ST elevation ratio to predict the morbidity of patients with inferior STEMI. Risk stratification had been assessed by other studies, presenting a 47% incidence of major in-hospital complications and found in-hospital morbidity to be increased in associations with RVMI [19,21]. A study involving patients with RVMI, showed a high frequency of VF in inferior STEMI with RVMI [2]. During this study, a transient AF developed in 7 (7.07%) patients, 7.3% patients had third-degree AV block for group 1 and 0.0% for group 2, and 8.8% of second-degree AV Mobitz II Block for group 1 and 0.0% for group 2. Inferior STEMI patients are uniquely susceptible to different types of heart block including Mobitz II AV block and third-degree heart block. There is a 10–20% incidence of high-degree heart block in inferior STEMI patients (21% for inferior MI with RVMI and 9.1% without); women and patients older than 70 years have a slightly increased incidence [22]. Serrano Jr and colleagues showed that 13% incidence of third-degree AV block and 5% for Mobitz II block on admission ECG in patients with inferior STEMI [23] with a higher rate of in-hospital mortality in inferior MI patients with heart block. Increased mortality could be the result of a larger infarct size rather than the consequence of heart block. Mortality rate was similar one year after hospital discharge. The onset of heart block may be variable from a progressive delay of conduction to the sudden development of third-degree heart block, and most patients will develop heart block within 24 hours of admission [22].

Cardiogenic shock was more frequent in group1patient (11.1%) than group 2 (1%). All patients with cardiogenic shock have RVMI, and there is a statistically significant association between cardiogenic shock and RVMI (p=0.0001). Recent studies have focused attention on the problem of cardiogenic shock associated with RVMI and have provided insights on the management and outcomes. A study showed an incidence rate of 6.9% in patients with inferior STEMI with RVMI and 5.5% without [2]. Alice Jacobs and colleagues reported a 5% rate of cardiogenic shock caused by RVMI [24].

Finally, in patients with RVMI complicating inferior STEMI, in-hospital PCI can reduce mortality compared with patients without RVMI even in those who treated with fibrinolytic therapy. So, the prognostic importance of ST elevation ratio in patients with inferior STEMI is of potential value to identify the patients with associated RVMI who are considered as a high-risk group and can benefit from an early invasive strategy [19].

Limitations of the study

The small sample size can affect the conclusions of the study. Furthermore, there is no reference investigation such as coronary angiography to validate the results of the study. The demographics of the patients showed a significantly higher prevalence of risk factors in group 1 patients which can be a contributing factor to the worse outcomes in those patients.

Conclusions

In patients with inferior STEMI, ST-segment elevation ratio in the lead III more than lead II can be a potential marker of the presence of RV infarction in association with inferior STEMI. Short-term prognosis is possibly worse in the presence of a higher ratio. However, further studies are needed to validate this conclusion.

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Author contribution

Dr Al-Mansouri and Dr Al-Obaidi: collection, analysis of data, interpretation and discussion of results done by both authors. Dr Al-Humrani: concept, supervision and revising the manuscript.

Conflict of interest

None to declare.

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The possible Association between Epstein-Barr Virus and Type 1 Diabetes Mellitus

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Abstract

Background Type-1-diabetes (T1D) also known as insulin-dependent diabetes mellitus (IDDM) or juvenile onset diabetes results from the progressive destruction of pancreatic beta cells resulting in insulin deficiency. Genetic factors are thought to be a major component for the development of T1D. The studies on the risk of developing T1D suggesting that the environmental factors, particularly viruses may be implicated in the initiation of beta cell destruction leading to T1D.

Objective To investigate the possible relationship between Epstein-Barr virus and T1D.

Methods The sera were collected from 56 T1D patients and 30 controls of age range 3-22 years old and from both sexes. The sera divided into two parts, one part for serology for detection anti-EBV EBNA-1 IgM and IgG antibodies by enzyme linked immunosorbent assay (ELISA) technique and the other for viral genomic extraction and conventional polymerase chain reaction (PCR) to detect the viral target gene.

Results The results by ELISA technique indicated that only 7 (12.5%) of T1D patients were positive for anti-EBV IgM antibody and 24 (42.9%) of T1D patients showed positive results for anti-EBV IgG antibody. In contrast, the control group showed negative results for both anti-EBV IgM and IgG antibodies. The results of PCR technique revealed that 15 (26.79%) of T1D patients have EBV DNA compared with none of the controls have EBV DNA (P<0.001).

Conclusion EBV infection may contribute to the pathogenesis or progression of T1D.

Keywords EBV, Type 1 Diabetes Mellitus, ELISA, PCR

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List of abbreviations: APC = Antigen presenting cell, CMV = Cytomegalovirus, EBV = Epstein-Barr virus, MHC = Major histocompatibility complex, T1D = Type 1 diabetes mellitus, T2D = Type 2 diabetes mellitus, TCR = T cell receptor

Introduction

Type 1 Diabetes Mellitus (T1D) is a rising worldwide health problem and the most common form of diabetes in childhood characterized by the body's inability to produce insulin due to selective loss of insulin-producing β-cells in the pancreatic islets [1,2]. Although the etiological factors for T1D are still obscure, epidemiological and genomic studies have been associated T1D with both environmental factors and genetic factors, i.e. polymorphisms in human leukocyte antigen (HLA) haplotypes [3,4].

It seems that viruses play a significant role among many environmental factors in the pathogenesis of T1D [5]. Most of the available data indicated that the viral infections are implicated in the development of T1D. A potential relationship between viruses and T1D is suggested by the evidence that some viruses can stimulate the disease in experimental animals and can be isolated from the pancreas of patients with newly diagnosed T1D [6-8].

Various viruses have been reported to be related with human T1D: Enteroviruses especially, Coxsackie B virus [9], Cytomegalovirus [10], Epstein-Barr virus, Rubella virus, Mumps virus, Rotavirus [11], and human Parvovirus [12].

Epstein–Barr Virus (EBV), also known as human herpes virus 4, is a γ-lymphotropic herpes virus and the causative agent of infectious mononucleosis (IM). The EBV genome is composed of a linear, double-stranded DNA with a relatively large genome size of approximately 180 kilobase pairs (kbp) that is encoded for many of the genes [13]. It was first identified in cells isolated from African Burkitt's lymphoma, later, it has been detected that it is highly prevalent around the world [14,15].

EBV has been associated with development of auto-immune diseases, this virus possesses a number of immune evasion mechanisms and immune-modulating proteins that make it a good candidate for initiation and progression of autoimmune diseases [16]. Therefore, it has been suggested that it is related to the development of T1D [17]. The mechanism by which EBV can contribute to the pathogenesis of T1D include the molecular mimicry. EBV infection may lead to cross-reactive autoimmune response through molecular mimicry between viral antigens and host proteins. A five amino acids-long sequence (GPPAA) in the Aspartic-57 region of the HLA-DQ8 β chain, which suggested to be important in defining the risk of T1D, is repeated 6 times in the BRF4-encoded EBNA3C protein of the EBV. Therefore, individuals who carry this sequence (GPPAA) in their HLA-DQ molecule present cross- reactivity to this epitope in EBV and may affect the pathogenesis of T1D [18]. The researchers found when EBV infects human immune cells, a protein produced by the virus -EBNA2- recruits human proteins called transcription factors to bind to areas of both the EBV genome and the cell's own genome. EBNA2 and its related transcription factors activate some of the human genes related to the risk for several autoimmune diseases, including T1D [19].

The objectives of this study were to detect the anti-EBV antibodies (IgG and IgM) in the sera of patients with T1D by using enzyme linked immunosorbent assay (ELISA), and to confirm the presence of EBV genome in the sera of patients with T1D using conventional polymerase chain reaction (PCR).

Methods

Patients and controls

This case-control study included 56 patients with T1D were collected from The Special Center of The Endocrine Glands and Diabetes in Al-Nassyrieh City during the duration from September to December 2018. The subjects were included 24 males and 32 females' patients with diagnostic features of T1D and age range was 3-22 years old. The diagnosis was based on the clinical and laboratory examinations. The second group is the control group, which included 30 apparently non-diabetic healthy people of males and females within the same age range of patient group. This study was approved by the Committee of Ethical Standards in the College of Science, Thi-Qar University and informed consent was obtained from all patients and controls before taking samples.

Five ml of fasting venous blood was taken from patients and controls. The blood was collected in coagulate gel tubes and centrifuged at 3000 rpm for 15 minutes to separate the serum. Each serum sample was separated in several 1.5 ml tubes stored at -20 ˚C for the serological and molecular tests.

Serological and Molecular study of EBV

Serological study of EBV

Detection of the serum level of the anti-EBV EBNA-1 IgM and IgG antibodies is based on the same principle of indirect ELISA assay using the EBV EBNA-1 IgM and EBNA-1 IgG antibody test kits from Demeditec/Germany, these kits are designed for the qualitative and the quantitative determination of specific IgM or IgG antibodies against EBV EBNA-1 in the

serum. The Microtiter strips are coated with EBV EBNA-1 antigen. Standards and diluted serum samples are pipetted into the wells of the Microtiter plate. The intensity of the color is directly proportional to the concentration of the IgM antibodies or IgG antibodies and measured at a wavelength of 450 nm.

Molecular study of EBV

The viral genomic DNA was extracted from serum samples by using the viral nucleic acid extraction kit from Geneaid/Taiwan, according to manufacturer's protocol.

The conventional PCR technique has been used in the current study for amplification the EBNA-1 gene of EBV DNA and the primers, which were used in this study were obtained by previous study. The PCR reaction mix consisted of 10 µl of extracted DNA, 2 µl of forward and reverse primers and 6µl distilled water. All these components were placed in the PCR tubes that contents all other components which needed to PCR reaction such as (Top DNA polymerase (1U), dNTPs, Reaction buffer (1x) with 1.5 mM $MgCl_2$, and stabilizer and tracking dye). The final volume per reaction tube was 20 µL. The PCR thermocycling conditions were done as shown in table (1). Then the PCR products were analyzed by 2% agarose gel electrophoresis and at molecular position 270 bp [20].

Table 1. Primer's sequence and PCR conditions for EBVNA-1 gene

Primer's sequence (5'- 3') of EBNA-1 gene	PCR conditions (Temperature (c) / Time)				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
		40 cycles			
F:GTCATCATCATCCGGGTCTC R:TTCGGGTTGGAACCTCCTTG	95/5min	95/30 sec	58/30 sec	72/30 sec	72/8min

Statistical analysis

The statistical analysis of this case-control study performed with the statistical package for social sciences (SPSS) 20.0 and Microsoft Excel 2013. Numerical data with normal distribution were described as mean and standard deviation, independent sample t-test used for comparison between two groups. Categorical data were described as count and percentage. Chi-square test or fisher exact test used to estimate the association between variables.

In order to measure the potential risk of pathogen in disease group, relative risk is a ratio of the probability of an event occurring in the exposed group versus the probability of the event occurring in the non-exposed group.

Results

Serological results

Serological results of the current study revealed that only 7 (12.5%) out of 56 T1D patients were positive for anti-EBV IgM antibody and the controls showed negative results for that type of antibody. Statistically, there was significant differences between the patient group and the control group (P = 0.043) in the presence of anti-EBV IgM. On the other hand, the results detected that (42.9%) of T1D patients were positive for anti-EBV IgG antibody compared with (0.0%) of the control group, indicating a highly significant difference (P < 0.001) as shown in tables (2) and (3).

Results of PCR

EBV genome was positive in 15/56 of T1D patients (26.76%) in the present study while the result of EBV genome detection was negative in controls. The results showed high significant difference (P <0.001) between T1D patients and controls in the EBV infection and the relative risk indicates to the positive relation between EBV and T1D as in table (4).

The positive results appeared in the molecular position about 270pb in gel electrophoresis and this position is a specific product size for EBNA-1 gene that used in this study to detect EBV infection (Fig. 1).

Table 2. Serological detection of anti-Epstein Barr virus IgM antibody in T1D patients and controls

			Study groups		Total
			T1D patients	Control	
Anti-EBV IgM	Positive	Count	7	0	7
		%	12.5%	0.00%	8.13%
	Negative	Count	49	30	79
		%	87.5%	100%	91.86%
Total		Count	56	30	86
		%	100%	100%	100%
P value				0.043	
Relative Risk with (CI)				1.612 (1.357-1.916)	

Table 3. Serological detection of anti-Epstein Barr virus IgG antibody in T1D patients and controls

			Study groups		Total
			T1D patients	Control	
Anti-EBV IgG	Positive	Count	24	0	24
		%	42.9%	0.00%	27.9%
	Negative	Count	32	30	62
		%	57.1%	100%	72.1%
Total		Count	56	30	86
		%	100%	100%	100%
P value				< 0.001	
Relative Risk with (CI)				1.938 (1.523-2.466)	

Table 4. Detection of the presence of EBV DNA by conventional PCR in T1D patients and control groups

			Study groups		Total
			T1D patients	Control	
EBV DNA	Positive	Count	15	0	15
		%	26.79%	0.0%	17.44%
	Negative	Count	41	30	71
		%	73.21%	100%	19.76%
Total		Count	56	30	86
		%	100%	100%	100%
P value			<0.001		
Relative Risk with (CI)			1.73 (1.42 - 2.11)		

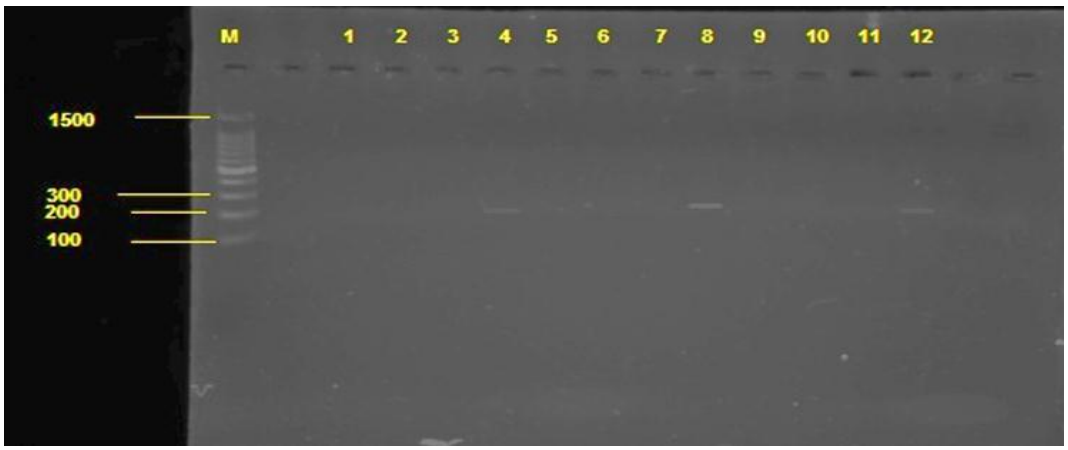

Figure 1. Detection of EBNA-1 gene for EBV by conventional PCR at molecular position 270bp, the positive result appeared in the samples (4, 8 and 12) in this image and other samples were negative

Discussion

T1D has a worldwide distribution with global variation in the incidence [21]. In Iraq, most of the e epidemiological studies do not distinguish between T1D and T2D in reporting the prevalence or incidence of the disease. However, there is a study reporting that the incidence of T1D in Basrah Province, southern part of Iraq was 7.4 per 100,000 during 2012-2016 [22]. EBV is one of the most common latent viruses inside the humans' B-lymphocytes and it has been documented as a causative agent of many cancers, where it has been shown that EBV infection strictly related to the malignant lymphomas in Iraq [23,24]. In addition, EBV may be considered a significant cause of renal impairment and kidney rejection in renal transplant patients [25].

The result of the current study showed presence of significant differences between T1D patients and control (P<0.001) in the detection of anti EBV IgG antibody. While regarding anti-EBV IgM antibody, the results of the current study also showed a significant difference between T1D patients and control (P= 0.043). Therefore, these findings might explain a positive correlation between the presence of anti-EBV IgM and IgG with T1D and

this probably suggested the role of the EBV infection in the pathogenesis of T1D.

Detection of antiviral antibodies, IgM or IgG refer to a viral infection then inflammation occurs. This inflammation has a role in the development and pathogenesis of T1D in different mechanisms. Many studies suggest that chronic infection result in increased processing and presentation of viral antigen which may have mimicry with host proteins. This mechanism involves a continuous acquisition of autoreactive events, leading to a chronic inflammatory state. This activation of multiple autoreactive T cells, as a result of tissue damage, is commonly referred to as epitope spreading and could be related to viral infections, by activating virus-specific T-cells or by direct virus-mediated self-tissue destruction. Virus-specific T cells become activated and migrate to the target tissues where they recognize the viral epitopes. The tissue destruction and release of self-antigens results in activation of autoreactive T cells and as a consequence an autoimmune response [26-28].

The inflammation that is induced by viral infection consists in the nonspecific activation of autoreactive T cells that have escaped thymic selection in a chronic inflammatory environment induced by viruses. Only a small fraction of activated T cells in viral infections are actually virus-specific. The others proliferate in the absence of the first signal (T cell receptor (TCR) + major histocompatibility complex (MHC) + Antigen). Cytokines, chemokines and other inflammatory mediators are secreted to promote a Th1 response, and increase the expression of MHC molecules, adhesion molecules and costimulatory molecules in the antigen presenting cells (APCs). The altered pattern of expression also affects the target cells, i.e. hyperexpression of MHC, adhesion molecules and antigen processing molecules among others. The release of self-antigens in the tissue and its presentation by macrophages or dendritic cells may prime virus-specific and autoreactive T cells. This effect has been observed by the transgenic expression of IFN-γ in insulin producing cells or oligodendrocytes resulting in the spontaneous development of diabetes or CNS demyelination respectively [29-32].

The results of PCR came to strengthen the possible association between EBV and T1D, especially our results showed significant differences ($P<0.001$) in the presence of EBV DNA between T1D patients and controls.

Detection of EBV by serology or by PCR gave evidence of the validity of the hypothesis that there is a relationship between EBV and T1D but the mechanism through which EBV might contribute to the pathogenesis of T1D remain uncertain. However, several possible scenarios can be included. First, EBV infection enhances immune cell cytotoxicity and tissue destruction through inducing the release of inflammatory cytokines. Second, EBV is resulting in local antiviral immune response that damage beta cells when the virus spread from B lymphocyte to pancreatic tissue [33,34]. Third, EBV infection may trigger a cross reactive autoimmune response through molecular mimicry of viral antigens and host proteins [18]. Other evidence showed that viral infections may play a role in accelerating the progression from beta cell autoimmunity to clinical T1D [35].

In conclusion, the findings of this study suggested EBV infection may have a role in the pathogenesis, development and progression of T1D.

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Author contribution

Dr. Mohammed: Idea, methods, and reviewing. Sabr: Materials, writing and publishing.

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Placental Alpha-Microglobulin 1 as A Marker of Preterm Prelabour Rupture of Membrane

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Abstract

Background Normal pregnancy requires that the physical integrity of the fetal membranes be maintained until term delivery.

Objective To detect preterm prelabour rupture of membrane in pregnant women with history of watery vaginal discharge by measurement of placental alpha microglobulin 1 in cervicovaginal fluid.

Methods A case-control study done at the department of Obstetrics and Gynecology of Al-Imamein Al-Kadhimein Medical City, included 100 pregnant women attending the Outpatient Clinic with a gestational age ranging between 28-36 weeks +6 days, 50 cases with rupture of membrane (study group) and 50 cases without any complaint (control group). All women underwent sterile speculum vaginal examination then nitrazine paper used, finally placental alpha microglobulin1 level was measured by using enzyme linked immunosorbent assay kit in vaginal washing fluid.

Results A highly significant association was found between mean of placental alpha microglobulin 1 in vaginal fluid of women with premature rupture of membrane compared to the control. The validity results of placental alpha microglobulin 1 findings regarding premature rupture of membrane include: sensitivity (100%), specificity (98.0%), +ve predictive value (98.1%), -ve predictive value (100%) and accuracy (99.0%), while for nitrazine; the sensitivity (94.0%), specificity (90.0%), +ve predictive value (90.4%), -ve predictive value (93.7%) and accuracy (92%) and for vaginal fluid sensitivity (80.0%), specificity (72.0%), +ve predictive value (74.1%), -ve predictive value (78.3%) and accuracy (76.0%).

Conclusion The placental alpha microglobulin-1 immunoassay in vaginal fluid wash found to be accurate and noninvasive test, in identifying preterm prelabour rupture of the membrane.

Keywords Placental alpha-microglobulin1, preterm prelabour rupture of membrane, prelabour rupture of membrane

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List of abbreviations: ARM = Artificial rupture of membrane, CDC = Centers for Disease Control and Prevention, FIGO = International Federation of Gynecology and Obstetrics, GA = Gestational age, IVH = Intraventricular hemorrhage, IGFBP-1 = Insulin like growth factor binding protein 1, PAMG-1 = Placenta-specific alpha microglobulin-1, PPROM = Preterm premature rupture of membrane, RDS = Respiratory distress syndrome, SD = Standard Deviation, SPSS= Statistical Package For Social Sciences, WHO = Word Health Organization

Introduction

Preterm premature rupture of membrane (PPROM) is responsible for nearly 40% of all preterm births [(1)]. Preterm birth, in turn, is a major global public health problem being responsible for 35% of the world's 3.1 million annual neonatal deaths. Prematurity is

the second largest direct cause of death in children less than 5 years [2,3].

The etiology of PPROM is multifactorial. Conditions that over distend the uterus, such as multiple gestation and polyhydramnios, may predispose to PPROM. Membranes that rupture prematurely may have different mechanical properties to those that do not rupture prematurely [4].

As well, the role of infection in the etiology of PPROM is clearly of great importance [4]. At term, programmed cell death and activation of catabolic enzymes, such as collagenase and mechanical forces, result in ruptured membranes. PPROM occurs probably due to the same mechanisms and premature activation of these pathways [5].

PPROM occurs from 24-36+6 weeks' gestation. Prematurity is the principal risk to the fetus, while infection morbidity and its complications are the primary maternal risks [5].

The major question regarding management of these patients is whether to allow them to enter labour spontaneously or to induce labor. Evidence supports the idea that induction of labor, as opposed to expectant management, decreases the risk of chorioamnionitis without increasing the cesarean delivery rate [6,7]. It is likely that multiple factors predispose certain patients to PPROM [8].

Diagnosis of PPROM is based on the history of vaginal loss of fluid and confirmation of amniotic fluid in the vagina. Episodic urinary incontinence, leucorrhea, or loss of the mucus plug must be ruled out [9]. A sterile vaginal speculum examination should be performed to confirm the diagnosis, to assess cervical dilation and length and to obtain cervical cultures and amniotic fluid samples for pulmonary maturation tests. On speculum examination, pooling of amniotic fluid in the posterior vaginal fornix can usually be seen [9].

Confirmation of the diagnosis can be made by: nitrazine paper test (amniotic fluid is mildly alkaline compared to normal vaginal secretions which are acidic) [10], fern test (after drying, amniotic fluid will form a crystallization pattern called arborization which resembles leaves of a fern plant when viewed under a microscope) [11], tampon test (using amniocentesis to inject dilute indigo carmine dye and looking for leaking of the blue fluid from the cervix onto a tampon) [12], ultrasonography (amniotic fluid index less than 5 cm is considered abnormal) [13], fetal fibronectin (fFN); a glycol protein present in amniotic fluid, placenta and the extracellular substance of decidua, may be normally detected in vaginal secretions up to 20 weeks gestation, and is then undetectable until about 36 weeks [13].

Several other markers have been studied for detection of PPROM including α-fetoprotein (AFP), insulin like growth factor binding protein-1 (IGFBP-1), prolactin, diamine oxidase activity, b-subunit of human chorionic gonadotropin (b-hCG) and placental α-microglobulin-1 (PAMG-1). However, results using such tests have been variable [14,15].

PAMG-1 is a human protein present in blood, amniotic fluid and cervicovaginal discharge of pregnant women. It is secreted from decidual part of the placenta and its concentration in the amniotic fluid is (2,000-25,000 ng/ml, which is several thousand magnitudes higher than that found in their background cervicovaginal discharge when the fetal membranes are intact (0.05-0.2 ng/ml) [16]. Further evidence demonstrated the efficiency of PAMG-1 to demonstrate the existence of injured membranes and leakage of amniotic fluid [17]. Cost benefit analysis was also shown to favor PAMG-1 over the traditional diagnostic methodology sequence [18]. The test is non-invasive, painless, covers the entire spectrum of pregnancy from week 16 to term, is specific for the presence of amniotic fluid and is of low cost. Results are available to the care giver in 5 min. The test is programmed to detect a minimum of 5ng/ml in the tested tissue. It is approved by the Federal Drug Administration (FDA) and is known commercially in the USA as Amnisure TM [19].

This study aimed to detect preterm prelabour rupture of membrane in pregnant women with

history of watery vaginal discharge by measurement of PAMG-1 in cervicovaginal fluid.

Methods

A case control study was conducted in the department of Obstetrics and Gynecology of Al-Imamein Al-Khadimein Medical City for the period from the 1st of February 2017 to the end of October 2017. It included 100 pregnant women attending the Outpatient's Clinic with gestational age ranging from 28-36 wks +6 days. Fifty pregnant women who had diagnosed with PPROM (the study group) and fifty pregnant women referred to obstetrics clinic for periodic check- up with no symptom or sign of rupture membrane (the control group). A verbal consent was obtained from them.

Inclusion criteria

Pregnant women with single viable fetus of gestational age 28-36 weeks +6 days confirmed by the first day of last menstrual period and first trimester ultrasound. Regarding the study group rupture of membranes was confirmed by examination using speculum and observation of cervical fluid leakage or accumulation of fluid in the posterior fornix of the vagina then nitrazine paper test after that ultrasound by specialist sonar doctor for amniotic fluid index.

Exclusion criteria

Congenital fetal malformations confirmed by U/S, fetal growth restriction, fetal distress at the time of presentation, patients with risk of ruptured membrane (diabetes mellitus, polyhydramnios, previous history of ruptured membrane), antepartum hemorrhage.

Samples collection and preparation

Patients were examined in semi recumbent position with good illumination using sterile Cusco speculum (without antiseptics), after waiting a few minutes to see if there is collection of fluid in the speculum.

This is augmented by asking the patient to cough allowing one to observe fluid escaping from the cervix. This means the pooling test is positive, after that we introduce nitrazine papers, which have yellow dye. This paper inserted in the posterior vaginal fornix for 15 seconds then if the color of the paper changes to blue color, the test should be considered as positive.

Measurement of vaginal fluid PAMG-1

In all patients 5 cc sterile normal saline was poured into the posterior fornix of the vagina by syringe 5 cc and after a few minutes the liquid was aspirated by the same syringe and was sent to the lab of the hospital to centrifuge it for 10 minutes and the enzyme linked immunosorbent assay (ELISA) kit of PAMG-1 were used to measure the concentration of PAMG-1.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 21 was used. Descriptive statistics presented as (mean ± standard deviation), frequencies & percentages. Chi-square used for categorical variables (Fishers exact test was used when expected variable was less than 20% of total). Independent sample t-test was used to compare between two means. Two by two tables were used to acquire the validity results of multiple tests in comparison to final outcome. ROC curve was used to assess the acceptable cutoff value. In all statistical analysis, level of significance (p value) set at ≤ 0.05.

Results

The mean age of the study group was 29.8±6.9 years and for the control was 29.7±6.6 years. No significant difference was observed between women with PPROM and those with no PPROM regarding women's age (p=0.1). Mean parity of women with PPROM was significantly higher than mean parity of women with no PPROM (p=0.01). A highly significant association was found between lower gestational age of pregnant women and PPROM (p<0.001). All these findings were shown in table 1.

Table 1. Distribution of demographic characteristics between the study and control groups

Variable		Study group		Control group		P value
		No.=50	%	No.=50	%	
Age	<20 years	9	18.0	2	4.0	0.1*NS
	20-29 years	14	28.0	20	40.0	
	≥30 years	27	54.0	28	56.0	
	Mean±SD	29.8±6.9		29.7±6.6		0.1*NS
Parity	Nulliparous	8	16.0	2	4.0	0.1***NS
	1-2 children	15	30.0	18	36.0	
	≥3 children	27	54.0	30	60.0	
	Mean±SD	3.5±2.6		2.5±1.3		0.01*S
Gestational age	28-33 weeks	43	86.0	24	48.0	<0.001*S
	34-36 weeks +6 days	7	14.0	26	52.0	
	Mean± SD	31.4±2.2		32.2±2.9		0.1*NS

*Chi square test, **Independent sample t-test, ***Fishers exact test, NS=Not significant (>0.05), S=Significant (≤0.05)

As shown in table 2, there was a highly significant association between mean of PAMG-1 in the women with PPROM than the women without PPROM (p<0.001).

Table 2. Distribution of PAMG-1 mean between the two groups

Variable	Study group Mean±SD	Control group Mean±SD	P value
PAMG-1 (ng/ml)	1259±378.6	64.2±231.7	<0.001*S

*Independent sample t-test, S=Significant (≤0.05)

There was a highly significant association between positive results of vaginal fluid test and PPROM women (p<0.001). A highly significant association was observed between positive results of nitrazine test and women with PPROM (p<0.001). The positive results of PAMG-1 were significantly associated with PPROM pregnant women (p<0.001). All these findings were shown in table 3.

Table 3. Distribution of diagnostic tests results between the two groups

Variable		Study group		Control group		P value
		No.=50	%	No.=50	%	
Vaginal fluid	Positive	40	80.0	14	28.0	<0.001*S
	Negative	10	20.0	36	72.0	
Nitrazine	Positive	47	94.0	5	10.0	<0.001*S
	Negative	3	6.0	45	90.0	
PAMG-1	Positive	50	100.0	1	2.0	<0.001**S
	Negative	0	0.0	49	98.0	

*Chi square test, **Fishers exact test, S= Significant

The validity results of PAMG-1 findings regarding PPROM were as follows, sensitivity (100%), specificity (98%), +ve predictive value (98.1%), -ve predictive value (100%) and accuracy (99%). All these findings were shown in table 4.

Table 4. Validity test results of PAMG-1 in the study groups

Validity test			Study group No. (%)	Control group No. (%)	Total No. (%)
PAMG-1	Positive	No. (%)	50 (98.1)	1 (1.9)	51 (100.0)
	Negative	No. (%)	0 (0.0)	49 (100.0)	49 (100.0)
	Total	No. (%)	50 (50.0)	50 (50.0)	100 (100.0)
Sensitivity			100%		
Specificity			98%		
+ve predictive value			98.1%		
-ve predictive value			100%		
Accuracy			99%		

The validity results of vaginal fluid pooling test findings regarding PPROM were as follows: sensitivity (80%), specificity (72%), +ve predictive value (74.1%), -ve predictive value (78.3%) and accuracy (76%). All these findings were shown in table 5.

Table 5. Validity test results of vaginal fluid pooling test in the study groups

Validity test			Study group No. (%)	Control group No. (%)	Total No. (%)
Vaginal fluid pooling test	Positive	No. (%)	40 (74.1)	14 (25.9)	54 (100)
	Negative	No. (%)	10 (21.7)	36 (78.3)	46 (100)
	Total	No. (%)	50 (50.0)	50 (50.0)	100 (100)
Sensitivity			80.0%		
Specificity			72.0%		
+ve predictive value			74.1%		
-ve predictive value			78.3%		
Accuracy			76.0%		

The validity results of nitrazine test findings regarding PPROM were as follows: sensitivity (94%), specificity (90%), +ve predictive value (90.4%), -ve predictive value (93.7%) and accuracy (92%). All these findings were shown in table 6.

The acceptable cut off points and the corresponding validity tests values PAMG-1 in prediction of PPROM from healthy pregnant women were shown in table 7 and figure1, cutoff PAMG level of >363.5 had acceptable validity results (100% sensitivity, 82.4% specificity, 100%PPV, 80.5% NPV and accuracy 94%).

Table 6. Validity test results of Nitrazine test findings in the study groups

Validity test			Study group No. (%)	Control group No. (%)	Total No. (%)
Nitrazine	Positive	No. (%)	47 (90.4)	5 (9.6)	52 (100.0)
	Negative	No. (%)	3 (6.3)	45 (93.7)	48 (100.0)
	Total	No. (%)	50 (50.0)	50 (50.0)	100 (100.0)
Sensitivity				94%	
Specificity				90%	
+ve predictive value				90.4%	
-ve predictive value				93.7%	
Accuracy				92%	

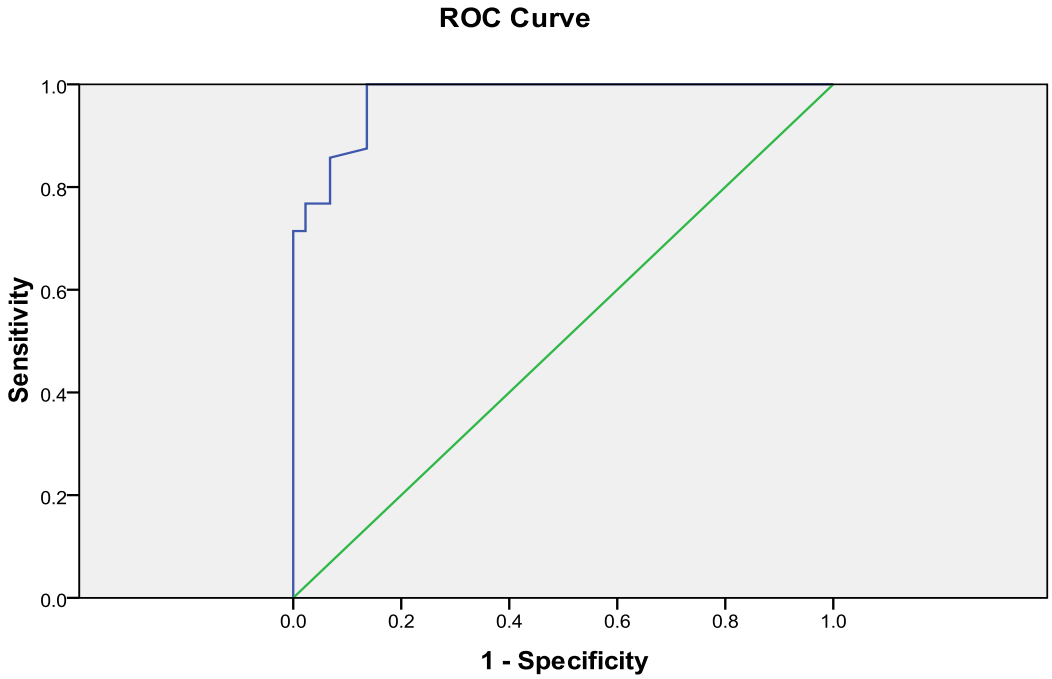

Figure 1. ROC curve for PAMG-1 prediction of PPROM (AUC=0.9)

Table 7. Coordinates of the ROC Curve of serum PAMG-1 in PPROM

Cutoff point	Sensitivity	Specificity	PPV	NPV	Accuracy
> 363.5	100%	82.4%	100%	80.5%	94%

Discussion

Unfortunately, there is absence of an accurate and simple diagnostic tool to establish the diagnosis as the traditional way to diagnose PPROM is subjective. However, each of these standard diagnostic methods was associated with high false positive or negative results [20]. Failure to genuinely exclude preterm PPROM in women presenting with symptoms of rupture of membranes could lead to unnecessary iatrogenic intervention resulting in delivery of

preterm babies with resultant problems of prematurity [21].

The present study revealed that there is no significant difference between the age and the PPROM but the significant association found with the gestational age which is in agreement to Lee et al., study in 2009 regarding women age but with no association was found with gestational age, and this may be due to differences in sample size collection [22].

Current study conclude that the (PAMG-1) test has high sensitivity, specificity, positive predictive value, negative predictive value and accuracy in the diagnosis of PPROM when its compared with other standard or usual clinical technique of assessment (Nitrazine test and vaginal fluid pooling test), and this is in agreement with that mentioned by Ng et al., 2013 [20], although in the current study the (PAMG-1) test was more accurate and had a better negative predictive value. In his study, the PMAG-1 had an overall sensitivity 97.5%, specificity of 100%, PPV of 100%, and NPV of 75.0% and the accuracy was 95.7%.

In a study done by Cousins et al. in 2005 [23], comparing between the AmniSure rapid immunoassay test (PAMG-1) with standard methods for diagnosing rupture of fetal membranes, found that the PAMG-1 is highly accurate with a sensitivity and specificity of 98% and 100%, respectively. Moreover, Lee et al., in 2007 [24], concluded that the PAMG-1 immunoassay test had significantly higher sensitivity compared to the combined conventional clinical tests, including speculum examination of fluid leakage, vaginal pooling, nitrazine and ferning tests, having sensitivity of 98.7% and 87%, respectively, but comparable specificity of 87.5% and 100%, respectively.

In a study of Yildiz et al., 2009 [25], PAMG-1 test confirmed PPROM with sensitivity of 85% and specificity of 100%, positive predictive value of 100% and negative predictive value of 87.5%, respectively. In comparison, those values for nitrazine test were 90.5%, 92.5%, 95.0%, 90.9%. Therefore, PAMG-1 immunoassay had an excellent specificity of 100% ($p > 0.05$) which is going with the results of our study.

Albayrak M et al. (26) 2011 found that the PAMG-1 test had sensitivity (97.4%), specificity (96.7%), positive predictive value (98.9%), negative predictive value (92.2%) and a diagnostic accuracy of (97.2%) which is less than our results finding that may be attributed to the different sample size.

This study concluded that he placental PAMG-1 immunoassay found to be quick, accurate and noninvasive, in identifying rupture of the membrane.

The current study recommends that PAMG-1 is cost effective and better predictor for detecting rupture of membrane when the diagnosis is in doubt.

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Author contribution

Dr. Seger: cases collection, obtaining the results of the tests used in the study. Dr. Al-Moayed, Dr. Abdulrasul and Dr. Mushatat: supervised the study and wrote the article and revised it.

Conflict of interest

The authors declare no conflict of interest for the present research outcome.

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Molecular Study of Biofilm Production by Methicillin Resistant *Staphylococcus aureus*

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Abstract

Background *Staphylococci* are a group of bacteria that cause diseases ranging from minor skin infections to life-threatening bacteremia. Biofilm formation was determined by a number of methods and is available to detect the capability of staphylococci to colonize the biomedical devices. The *icaA* and *icaD* have been reported to play a significant role in biofilm formation.

Objective To achieve and detect the molecular basis of adhesion properties in respect to methicillin resistant *Staphylococcus aureus*.

Methods Clinical samples were taken from Burn patients; identified and Methicillin susceptibility was tested. The genes *icaA* and *icaD* were amplified in methicillin resistant *Staphylococcus aureus* and the polymerase chain reaction products were sequenced and aligned with the previous recorded sequences online.

Results There was a great correlation between the presence of *icaD* genes and the slime production. Methicillin resistant *Staphylococcus aureus* did not reveal any correlation between *icaA* and *icaD* and slime layer production; nonetheless, a correlation was noticed between *icaD* alone and a biofilm production

Conclusion The present findings indicated that methicillin resistant *Staphylococcus aureus* was able to form biofilm. None of the methicillin resistant *Staphylococcus aureus* isolates harboured *icaA*; while 100% of them contained *icaD*.

Keywords Methicillin resistant *Staphylococcus aureus*, *icaA*, *icaD* gene

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List of abbreviations: CRA = Congo Red Agar, DNA = Deoxy nucleic acid, MRSA = Methicillin resistance Staphylococcus aureus, MRSE = Methicillin resistance staphylococcus epidermidis, MtP = Microtiter plate method, OD = Optical density

Introduction

Staphylococci are a diverse group of bacteria that cause diseases ranging from minor skin infections to life-threatening bacteremia. In spite of large-scale efforts to control their spread, they persist as a major cause of both hospital and community acquired infections worldwide. The two major opportunistic pathogens of this genus are *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) [(1)]. The widespread use of Methicillin and other semisynthetic penicillin in the late 1960s led to the emergence of Methicillin resistance *S. aureus* (MRSA) and *S. epidermidis* (MRSE), which continue to persist in both the healthcare and community environments. Biofilm formation may be determined by a number of available methods determine the capability of *staphylococci* to colonize the biomedical catheters. The Congo red agar (CRA) assay described by Freeman et al. [(2)] and/or the microtiter plate (MtP) test devised

by Christensen et al. [3] were the most commonly used as the phenotypic methods for the detection of biofilm production. The *icaA* and *icaD* have been reported to a play a significant role in biofilm formation. The *icaA* gene encodes N acetyl glucosaminyl transferase, the enzyme involved in Polysaccharide intercellular adhesion (PIA) synthesis. On the other hand, *icaD* has been reported to a play a critical role in the maximal expression of N-acetylglucosaminyl transferase, leading to the full phenotypic expression of the capsular polysaccharide [4]. Wide controversial aspects were emerged about the nature of MRSA and MRSE biofilms, the basis of adhesion and best method for detection. From this perspective, the present study was designed and aimed to achieve to achieve and detect the molecular basis of adhesion properties in respect to methicillin resistant *S. aureus* by evaluating the most frequent methods (CRA and MtP) employed for the detection of adhesion properties in respect to MRSA and MRSE, detecting the presence of the icaA and icaD in MRSA and MRSE isolates and finally determination of the nature of biofilm adhesion via treatment with proteinase K and $NaIO_4$.

Methods

Specimen

Fifty clinical specimens referring to burn were collected from patients attending Sulaimani Teaching Hospital, Emergency Hospital, and Child Teaching Hospital; for the period from November 2018 to March 2019. The specimens were collected by the attending physician and health officer using sterile applicator stick with cotton swabs moistened with normal saline and test tubes were used to collect the sample. Bacteria were stored for more than three months in nutrient broth containing 20% glycerol at (-20 ˚C) without significant loss of viability.

Isolation of *staphylococci*

All specimens were streaked on mannitol salt agar and blood agar. Thereafter, all plates were incubated aerobically for 24 h at 37 °C. Isolates were identified by the Vitek system.

Biofilm formation by microtiter plate method (MtP)

A suspension of bacterial isolate that equivalent to the McFarland No. 0.5 turbidity standard were inoculated in Nutrient broth and incubated for 18-24 h at 37 ˚C in individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plate stationary phase. Nutrient broth culture supplemented with glucose (0.5%) or NaCl (1%). After that, 200 µl of the inoculum were transferred to the assay wells, which corresponds to an inoculum of approximately 5×10^6 cells/well. Subsequently, inoculated assay plates were transferred to an incubator set at 37 °C for 18-24 h without shaking. Negative and positive control wells were included in the test. After incubation, the optical density (OD) was measured by spectrophotometer at OD 570 nm of each well using a multi-well plate reader to quantify overall growth (Table 1).

Genomic DNA extraction and amplification of icaA and icaD genes

Genomic DNA from all biofilm producer isolates (37 MRSA) was extracted using Genomic DNA Extraction kit (Promega, USA), then the presence of the *icaA* and *icaD* genes these isolates were detected as described by Arciola et al. [5], with two sets of primers for *icaA* F5'-TCTCTTGCAGGAGCAATCAA-'3 and *icaA* R5'TCAGGCACTAACATCCAGCA-'3, for *icaD* detection F5'-ATGGTCAAGCCCAGACAGAG-'3 and *icaD* R5'-CGTGTTTTCAACATTTAATGCAA-'3. Reaction conditions were 94 °C for 5 min initial incubation, 94 °C for 30 sec denaturation, 55.5 °C for 30 sec annealing, 72 °C for 30 sec extension and final extension for 1 min at 72 °C.

DNA Sequencing

Purified PCR products were sent to Macrogen Company, Korea for the DNA sequencing and analyzed by NCBI Blast tools.

Results

Isolation and Identification

Of *Staphylococci* from collected samples, only 50 isolates (91%) have grown on Mannitol salt agar [6]. Taking together, the results were revealed that all 37 isolates were diagnosed as *S. aureus*; whereas the other 13 were comprised as *S. epidermidis*.

Biofilm detection by microtiter plate method (MtP):

The present findings indicated that MRSA was able to form biofilm, and the (OD) value ranged between 0.147-0.315. Using MtP method for the detection of biofilm formation *S. aureus* isolates, when grown in nutrient broth without any supplementation, 100% MRSA isolates were able to form weak biofilm (Table 1).

Table 1. Classification of bacterial adherence by micro titer plate method

Mean OD750	Adherence Biofilm Formation
OD ≤ ODc	Non-adherent
ODc < OD ≤ 2*ODc	Weakly adherent
2*ODc < OD ≤ 4*ODc	Moderately adherent
4*ODc < OD	Strongly adherent

Amplification of *icaA* and *icaD* genes

PCR amplicons obtained from genomic DNA extracted from Positive control MRSA isolate yielded a 188-bp band for *icaA*, and a 198-bp band for *icaD* genes (figure 1). Results of PCR study for 37 genomic DNA extracted from MRSA isolates revealed that 0/37 (0%) MRSA isolates had *icaA* gene, while 37/37 (100%) harbored *icaD*. The current results, suggests that all MRSA isolated from burn specimens were *icaD* positive (figure 1).

DNA sequencing

In order to confirm the results of *icaA* and *icaD* amplification, PCR products were sequenced, analyzed by Bio-Edit software and similarity searches were carried out using with the Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Results revealed that GenBank accession numbers for the nucleotide sequences of the *icaA* gene fragments were reference isolates DQ846812, DQ846811, and DQ836167 whereas those of *icaD* gene fragments were AY138959 and FN433596. However, some deletions and insertions of nucleotides were noticed (Figure 2).

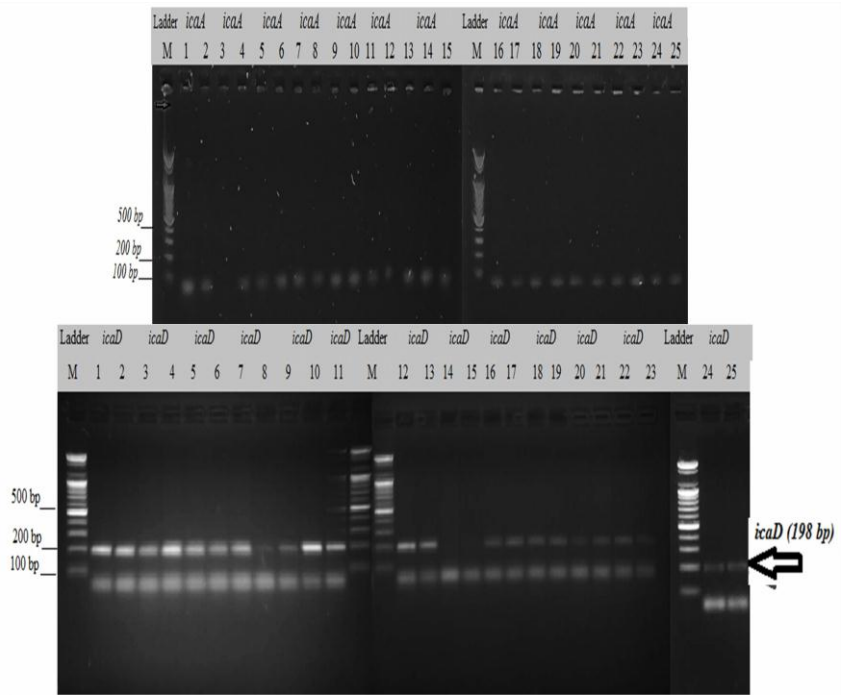
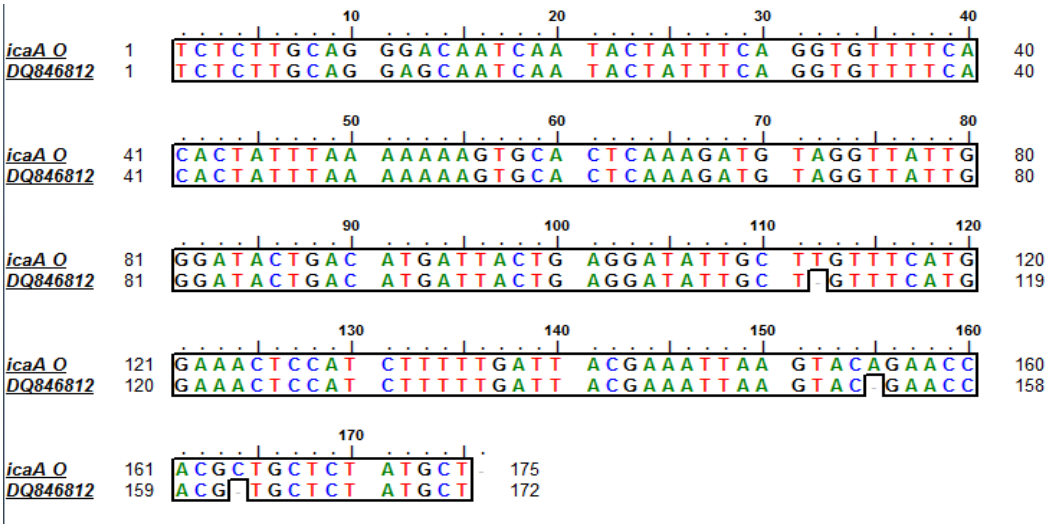


Figure 1. Agarose gel electrophoresis of polymerase chain reaction amplification of *icaA* and *icaD* genes in methicillin resistant *S. aureus* (numerals). M represents 100 bp DNA molecular size marker, in 1.6 % Agarose gel on (85 V for 90 minute). Visualized under U.V light after staining with Ethidium bromide dye

Discussion

Babakir-Mina et al. [7] stated that *S. aureus* accounted for 22% of all patients in Sulaymaniyah Burn Hospital, and constituted 36% from burn specimens. Resistance to methicillin in *Staphylococcus spp*. is primarily mediated by the presence of penicillin-binding protein 2a, encoded by the *mecA* gene. In certain MRSA strains, the *mecA* gene is heterogeneously expressed in vitro [8]. Locally, according to the results of Al-Dahbi [9], the incidence of MRSA among *S. aureus* was 94.3%, Babakir-Mina [7] observed that among *S. aureus* positive cases, 88% were MRSA. Bacteria isolates from burn infection seems to be more resistant to most other antibiotics compared to other sites. Sputum seemed to have the lowest Methicillin resistance percentage in comparison to other specimens. Cefoxitin is a cephamycin antibiotic and has been described as an inducer of methicillin resistance [10]. The performance of cefoxitin either as a disc or as a supplement in agar medium for the detection of MRSA has been confirmed extensively [11]. According to the literature, the quantitative MtP assay eliminates subjectivity in reading of obtained results and predicts clinical relevance more reliably than the tube test [12]. This method has been reported to be the most sensitive, accurate and reproducible screening method for determination of biofilm production by clinical isolates of staphylococci and has the advantage of being a quantitative tool for comparing the adherence of different strains [13]. The *icaA* operon genes have been widely described in *S. epidermidis* and *S. aureus*, several authors have found similarity in other coagulase negative staphylococci species. Nevertheless, results cannot be extended to all pathogenic species [12]. As it is reported by these authors, the genes of *ica* operon frequently appeared in strains of *S. aureus* [14].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DQ846812 41 CACTATTTAA AAAAAGTGCA CTCAAAGATG TAGGTTATTG 80
icaA O 81 GGATACTGAC ATGATTACTG AGGATATTGC TTGTTTCATG 120
DQ846812 81 GGATACTGAC ATGATTACTG AGGATATTGC TGTTTCATG 119
icaA O 121 GAAACTCCAT CTTTTTGATT ACGAAATTAA GTACAGAACC 160
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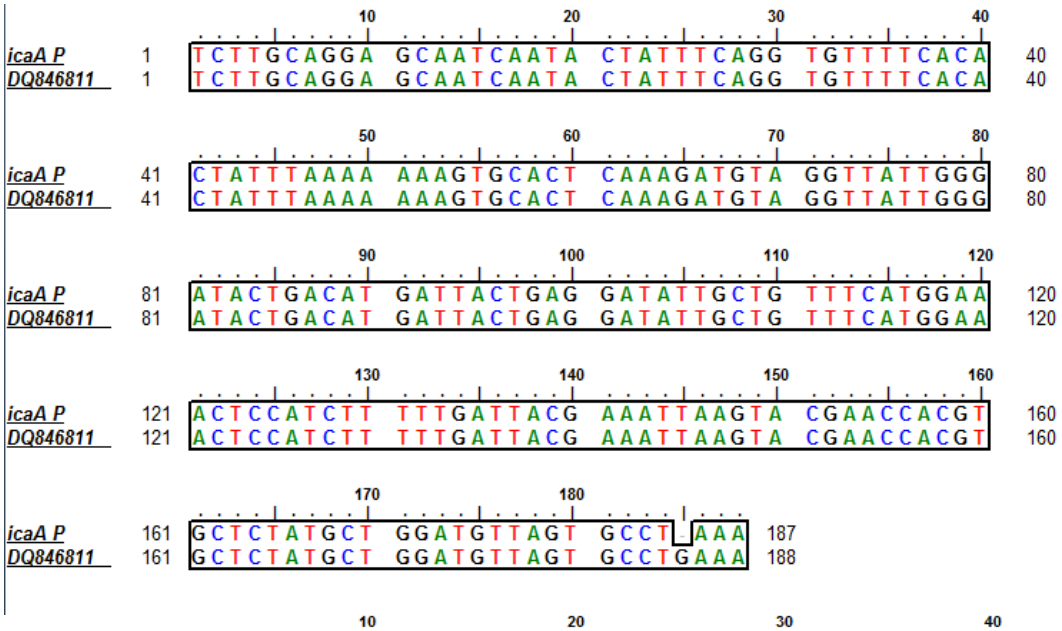
DQ846812 159 ACGTGCTCT ATGCT 172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DQ846811 41 CTATTTAAAA AAAGTGCACT CAAAGATGTA GGTTATTGGG 80
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DQ846811 121 ACTCCATCTT TTTGATTACG AAATTAAGTA CGAACCACGT 160
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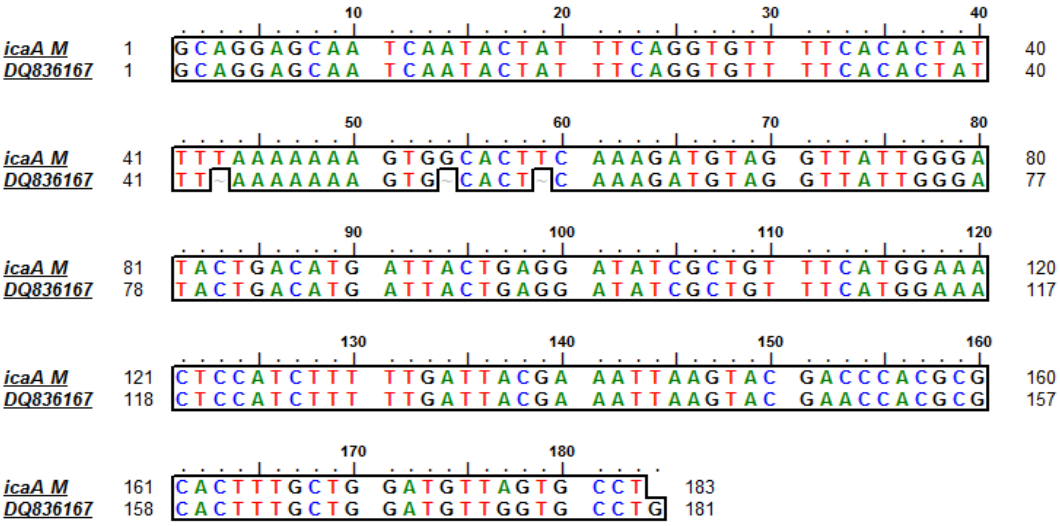
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DQ836167 41 TTAAAAAAA GTGCACTC AAAGATGTAG GTTATTGGGA 77
icaA M 81 TACTGACATG ATTACTGAGG ATATCGCTGT TTCATGGAAA 120
DQ836167 78 TACTGACATG ATTACTGAGG ATATCGCTGT TTCATGGAAA 117
icaA M 121 CTCCATCTTT TTGATTACGA AATTAAGTAC GACCCACGCG 160
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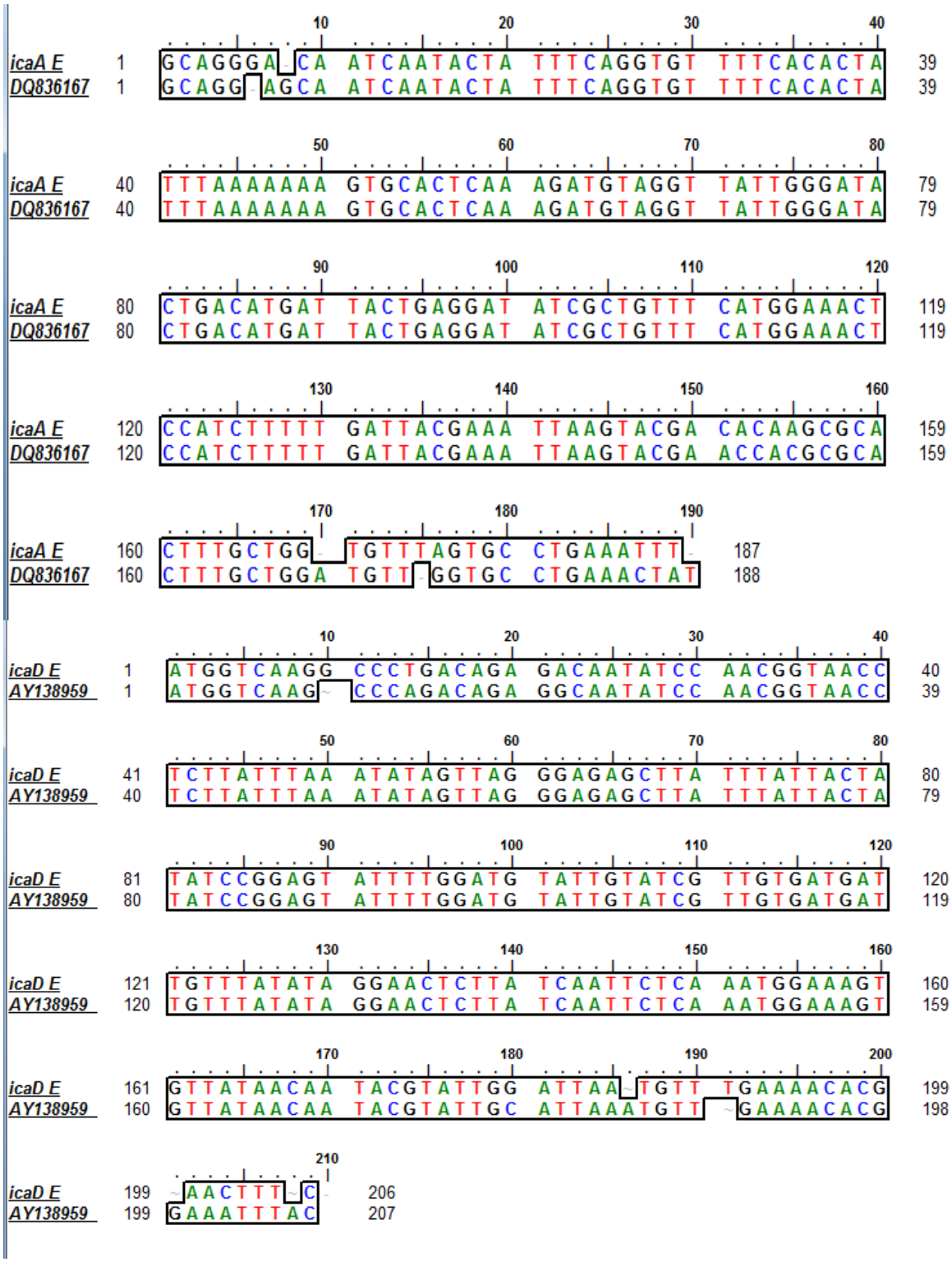
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DQ836167 120 CCATCTTTTT GATTACGAAA TTAAGTACGA ACCACGCGCA 159
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DQ836167 160 CTTTGCTGGA TGTT-GGTGC CTGAAACTAT 188
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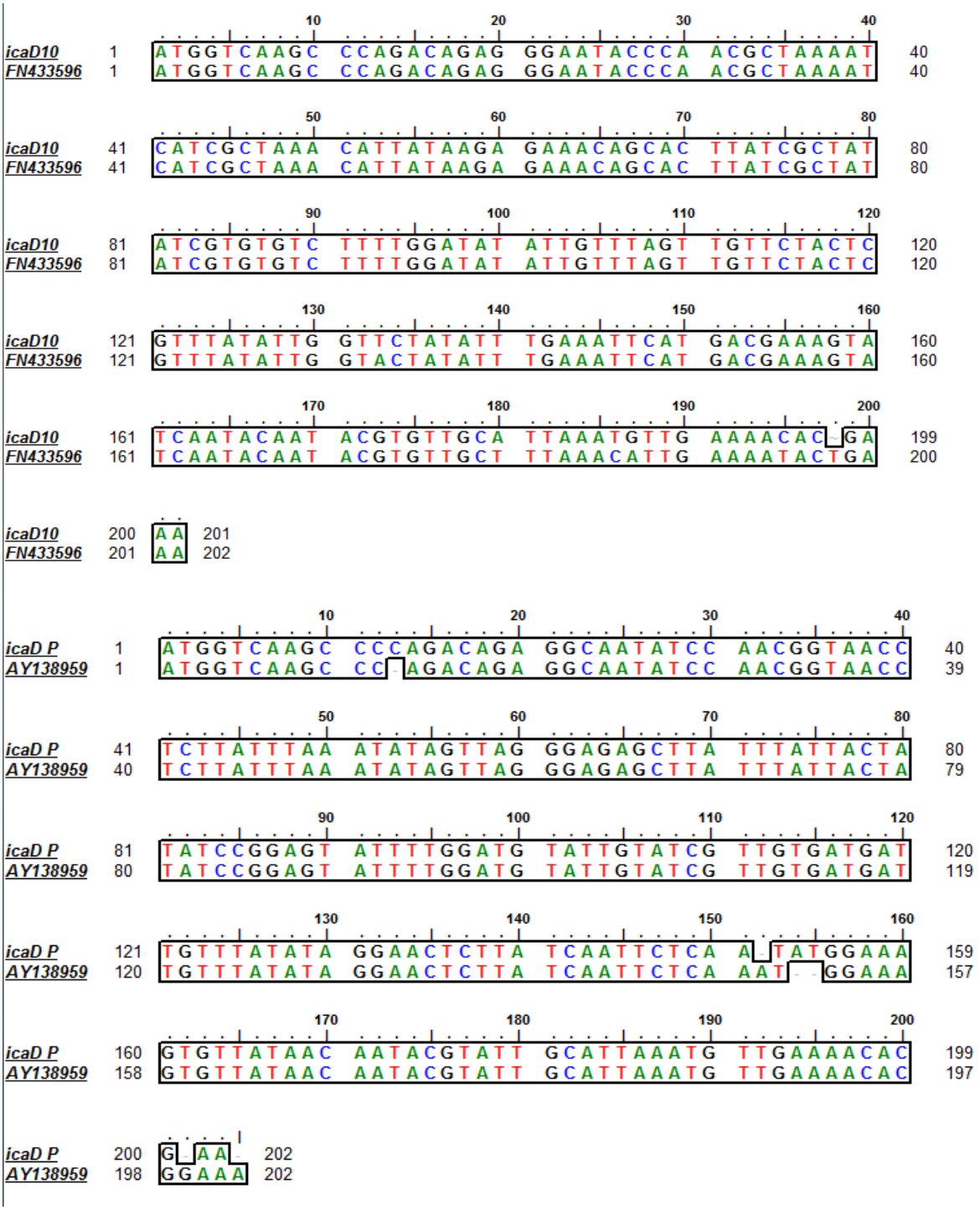
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FN433596 161 TCAATACAAT ACGTGTTGCT TTAAACATTG AAAATACTGA 200
icaD10 200 AA 201
FN433596 201 AA 202
icaD P 1 ATGGTCAAGC CCCAGACAGA GGCAATATCC AACGGTAACC 40
AY138959 1 ATGGTCAAGC CC-AGACAGA GGCAATATCC AACGGTAACC 39
icaD P 41 TCTTATTTAA ATATAGTTAG GGAGAGCTTA TTTATTACTA 80
AY138959 40 TCTTATTTAA ATATAGTTAG GGAGAGCTTA TTTATTACTA 79
icaD P 81 TATCCGGAGT ATTTTGGATG TATTGTATCG TTGTGATGAT 120
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icaD P 121 TGTTTATATA GGAACTCTTA TCAATTCTCA A-TATGGAAA 159
AY138959 120 TGTTTATATA GGAACTCTTA TCAATTCTCA AAT--GGAAA 157
icaD P 160 GTGTTATAAC AATACGTATT GCATTAAAATG TTGAAAACAC 199
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icaD P 200 G-AA 202
AY138959 198 GGAAA 202

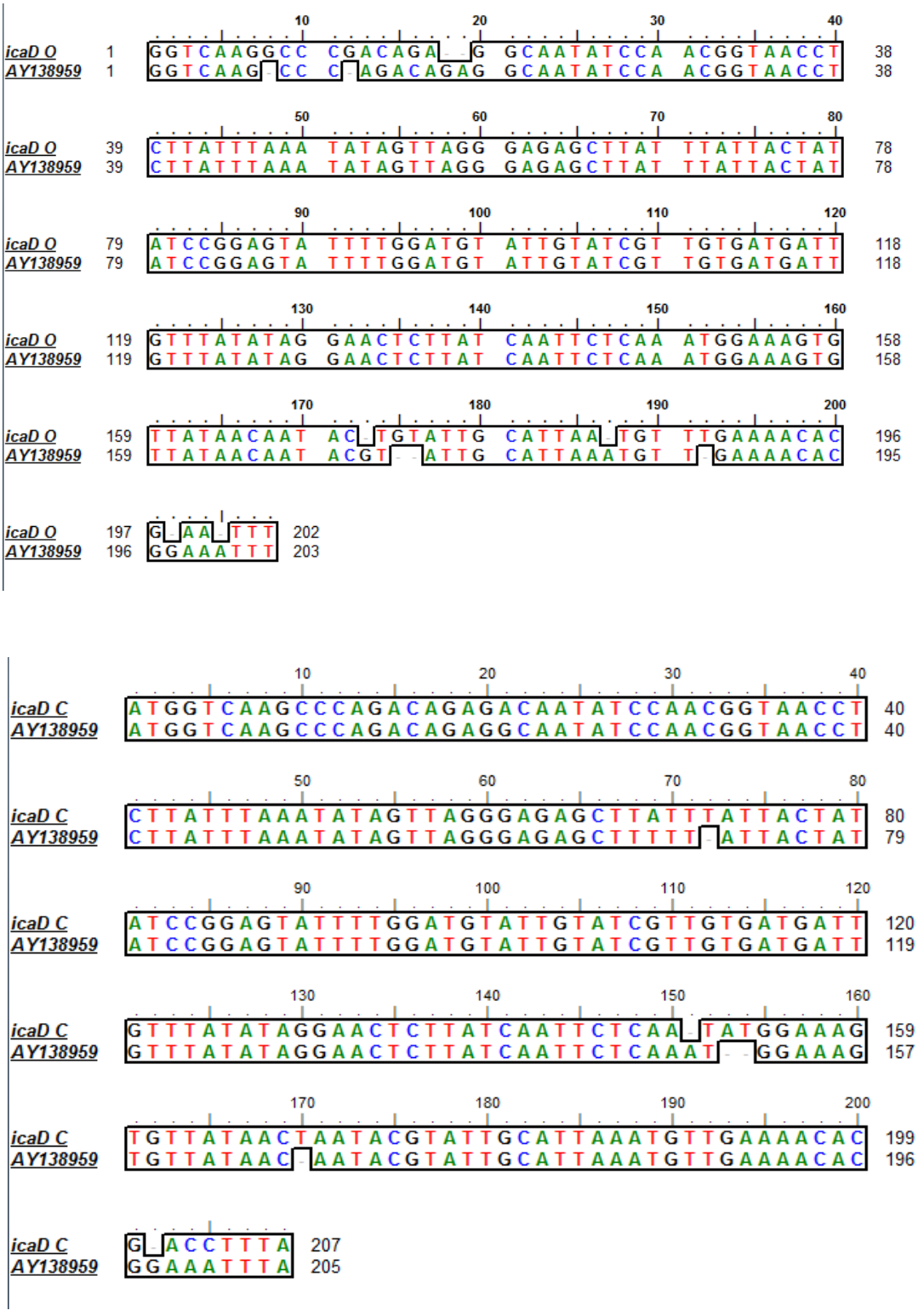

Figure 2. Multiple sequence alignment of nucleotide sequencing *Staphylococcus aureus* clinical isolates in Sulaymaniyah hospitals. Numbers beside the gene names represent MRSA. The codes below the gene name signify the accession number

The obtained results have an agreement with those of Petrelli et al. *(15)* as they recorded the existence of the *icaA* and *icaD* genes in about 94.6% contained both *icaA* and *icaD*. In contrast to the current results, when as the finding in the current study that all MRSA isolated from burn specimens were icaD positive. Diemond-Hernandez et al. [16] reported that *icaA* genes were present in 27.8%, of coagulase negative staphylococci isolates and only (10%) of *S. aureus* isolates were positive for *icaA* + *icaD* genes. Zhou et al. [17] demonstrated that *icaD* had higher positive rate than *icaA* in all *S. aureus* isolates. Other findings pointed to an important role of the *icaA* and *icaD* due to their ability to produce slime strongly in a high percentage of clinical isolates collected from patients with catheters associated infection [18]. Zhou et al. [17] reported that the co-expression of *icaA* with *icaD* can increase slime production remarkably.

From the present study it can be concluded that all MRSA isolates have the ability to produce a slime layer in different amounts of production. This study indicates the absence of *icaA* from the genome of MRSA isolates; whereas, most of MRSA harbored *icaD* gene.

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Conflict of interest

The author has no conflict of interst.

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Detection of ETV6/RUNX1 Fusion Gene Using FISH Technique Detection in Pediatric ALL patients

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Abstract

Background One of the commonest genetic subtypes of acute lymphoblastic leukemia (ALL) is t (12;21) (ETV6/RUNX1) being associated with favorable prognosis and distinctive clinical and pathological features. There are few studies about this abnormality in Iraq.

Objective To detect the expression ETV6/RUNX1 fusion gene in B-ALL pediatric patients by using FISH technique.

Methods This cross-sectional study was conducted from April 2018 to September 2018. Forty-eight newly diagnosed children with B-ALL were enrolled in this study. Fresh peripheral heparinized blood sample (3 ml) were taken from the patient at admission before chemotherapy, and ETV6-RUNX1 probe was applied and reading done by florescent microscope.

Results The mean age of study group was (4.01±0.19) years, their median age was 4.1 years, ranging between (2-7.2) years at diagnosis, ETV6/RUNX1 chimeric transcript product was found in 19 of 48 (39.6%) pediatric B- ALL patients.

Conclusion The frequency of investigated translocation [t(12;21)/ETV6/RUNX1 in a sample of Iraqi pediatric B-ALL patients, was among the higher reported frequencies worldwide, and that ETV6/RUNX1 fusion gene is independent prognostic factor not related to other hematological and clinical parameters.

Keywords ETV6/RUNX1 fusion gene, pediatric ALL, FISH

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List of abbreviations: ALL = Acute lymphoblastic leukemia, FISH = Fluorescent in-situ hybridization, FTA cards = Flinders technology associate cards, Hb = Hemoglobin, LDH = Lactate dehydrogenase, PCR = Polymerase chain reaction, RBC = Red blood cell, WBC = White blood cell

Introduction

Genetic studies in acute lymphoblastic leukemia (ALL) had been a major contributing factor in diagnosis, prognosis therapy and shedding lights on the pathogenesis of the disease [(1)]. Therefore, classification is very important in ALL diagnosis. The six common genetic subtypes of ALL are t(1;19)(E2A-PBX1), t(12;21)(ETV6-RUNX1), t(9;22)(BCR-ABL), t(4;11) *MLL*-rearrangement and hyperdiploidy [(2)].

In 1995, two research teams discovered the t(12;21)(p13;q22) translocation, followed by other studies demonstrating that it is the most common genetic abnormality in pediatric ALL constituting about 25% of pediatric B-ALL while it was less frequent in adult ALL constituting nearly 2% [(3)].

However, by applying conventional cytogenetics, this chromosomal abnormality is barely detectable and may occur in less than 0.05% of childhood ALL. This is because the

t(12;21) is usually cryptic, and involve portions of the two chromosomes that are both small and have similar banding patterns. Therefore, it is better to be detected by fluorescence in situ hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR) [4-6].

The ETV6-RUNX1 fusion gene may arise as an early event during the prenatal period in pediatric ALL. This led to emergence of pre-leukemic clone, which after birth may give rise at low frequency to ALL after the having another necessary secondary genetic abnormality [7].

This work was done to detect the expression of ETV6/RUNX1 fusion gene in B-ALL pediatric patients by using FISH technique, and to find the correlation of ETV6/RUNX1 fusion gene to hematopathological parameters including complete blood count finding, blast count and lactate dehydrogenase (LDH).

Methods

A cross sectional study was conducted on 48 newly diagnosed B-ALL patients, who were attending Children Well Fair Teaching Hospital from April 2018 to September 2018.

The diagnosis of B-ALL depended on clinical findings, morphology and immunophenotype. The patient clinical data was obtained from patient hospital record and clinical monitoring chart.

After taking informed written consent from one or both parent, patients' samples were taken at admission. Peripheral blood collected in Na^+ heparinized tube, 1 ml of Na^+ heparinized blood sample labeled with patient name, age and date and those were stored as a fixed pellet at 4 ^oC in methanol: acetic acid (3:1) until FISH studies performed.

FISH was performed using directly labeled ETV6/RUNX1 Dual Fusion probes (Metasystem D-5115-100-OG) to show ETV6/RUNX1 fusion gene signals in cells with t (12:21) on chromosomes 21.

The orange labelled probe spans the breakpoint at 21q22(RUNX1) (646Kb) and include DNA sequence that hybridize (21q22.1), while the green labelled probe spans the breakpoint at 12p13(ETV6) (448Kb), which had DNA Sequence that hybridize (12p13).

Preparation of uncultured blood and slide preparation was done by applying standard protocol [8].

Slide reading was done by meta system fluorescents microscope using strict scoring criteria for FISH, orange RUNX1 signals are referred to as O, green ETV6 signals are referred to G, and ETV6/RUNX1 fusion signals as yellow infuse with green and orange. For each specimen, each microscopic scored 500 consecutive qualifying interphase nuclei from different area of the same slide. Samples were considered translocated positive when 4% cell showed the presence of fusion nuclei in which two probes were fused.

Statistical analysis

The collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 25. p value of >0.05 was considering to be non-significant, <0.05 was consider to be significant.

Results

The mean age of the study group was (4.01±0.19) years (mean±SE), their median age was 4.1 years, ranging between (2-7.2) years at diagnosis, majority of patient were 3 and 4 years old. Among 48 patients, 18 were females, representing (37.5%) and 30 were males representing (62.5%).

ETV6/RUNX1 fusion gene expression was positive in 19 patients representing (39.6%). While it was negative in 29 patients representing (60.4%) (Figures 1 and 2).

Regarding gender distribution (Table 1), 16 (53.3%) male patients were negative and 14 (46.7%) male patients were positive for the fusion gene, regarding female patients; 13 (72.2%) were negative and 5 (27.8%) were positive (P = 0.2) for the fusion gene. There was no significant difference in relation to gender between ETV6/RUNX1 positive cases and ETV6/RUNX1 negative cases (P = 0.20).

WBC count was significantly higher in ETV6/RUNX1 positive cases than in negative

cases, while hemoglobin level, platelet count, blast percent and LDH level showed no significant difference between positive and negative cases (table 2).

There was no significant difference in regard to clinical feature between ETV6/RUNX1 positive and negative groups (table 3).

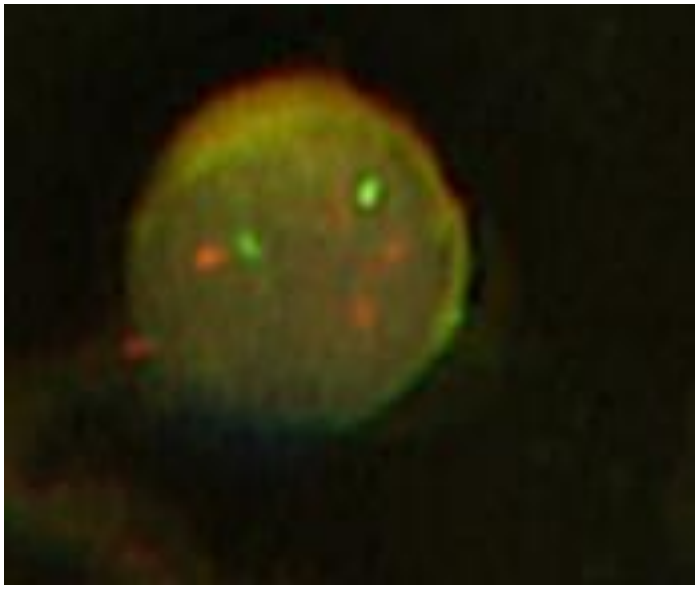

Figure 1. Fluorescent microscope image showing normal cells without fusion gene expression, 2 green signals for ETV6 gene, 2 orange signal for RUNX1 gene

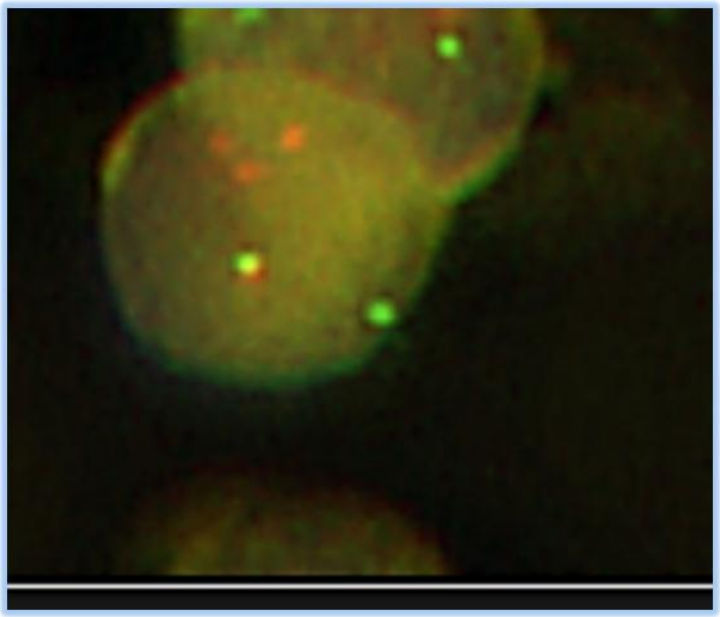

Figure 2. Fluorescent microscope image showing ETV6/RUNX1 fusion gene (green, orange and yellow)

Table 1. ETV6/RUNX1 fusion gene expression in relation to gender

Parameter	Variable	Male (n=30)	Female (n=18)	P
ETV6/Runx1	-ve N (%)	16 (53.3%)	13 (72.2%)	0.20 *NS
	+ve N (%)	14 (46.7%)	5 (27.8%)	

* Chi square test, NS: Non-Significant (P>0.05)

Table 2. White blood cell, hemoglobin, Platelet count and Lactate dehydrogenase in relation to ETV6/RUNX1 fusion gene expression

Variable		-ve N=29	+ve N=19	P
WBCs (10^9/L)	Mean±SE	39.92±4.83	60.72±8.11	0.04 *
	Median (Range)	35 (2.8-96)	69 (2.5-133)	S
Hb (mg/dl)	Mean±SE	9.54±0.27	9.19±0.34	0.42 *
	Median (Range)	9.4 (6.8-12.3)	9.2 (7.1-12.2)	NS
Platelets (10^9/L)	Mean±SE	129.14±10.62	120.95±10.45	0.84 *
	Median (Range)	111 (65-280)	110 (51-200)	NS
Blast cell (%)	Mean±SE	65.0±4.79	67.05±5.99	P=70 *
	Median (Range)	72 (14-95)	79 (11-95)	NS
LDH (IU)	Mean±SE	948.34±78.27	1033.2±92.55	0.49 *
	Median (Range)	989 (110-1882)	1110 (156-1781)	NS

*Mann Whitney test *significant (p<0.05), NS: Non-Significant (P>0.05)

Table 3. Clinical features in relation to ETV6/RUNX1 fusion gene expression

Symptoms and signs	-ve N=29 N (%)	+ve N=19 N (%)	P
Fever	19 (65.5%)	12 (63.2%)	0.87 * NS
Hepatosplenomegaly	14 (48.3%)	10 (52.6%)	0.77 * NS
Pallor	12 (41.4%)	8 (42.1%)	0.96 * NS
Vomiting	13 (44.8%)	5 (26.3%)	0.20 * NS
Weight loss	5 (17.2%)	4 (21.1%)	0.74 * NS
Jaundice	3 (10.3%)	4 (21.1%)	0.31 * NS
Lymphadenopathy	2 (6.9%)	2 (10.5%)	0.66 * NS
Nausea	2 (6.9%)	2 (10.5%)	0.66 * NS
Anorexia	2 (6.9%)	2 (10.5%)	0.66 * NS
Lethargy	3 (10.3%)	0 (0.0%)	0.15 * NS
CNS involvement	2 (6.9%)	0 (0.0%)	0.24 * NS
Bone pain	0 (0.0%)	1 (5.3%)	0.21 * NS

* Chi square test, NS: Non-Significant (P>0.05)

Discussion

In this study, t(12;21)/ETV/RUNX1 was detected by using FISH technique in 19/48 patient representing (39.6%). In Iraq, focus on these translocation done by two studies dealing with ETV6/RUNX1 fusion gene expression, Salih study that was done at 2015 using RT-PCR on 47 children to evaluate different types of translocation in ALL, revealed the presence of molecular abnormalities in (51.06%) patients; (27.65%) had ETV6/RUNX1, Other study done by Al-Kzayer et al. during 2012 using flinders technology associate (FTA) card on Iraqi children and it was conducted in Japan where ETV6/RUNX1fusion gene was detected in (12.1%) [9,10].

The result of current study goes with other study that showed the ETV/RUNX1 is the most frequent translocation of ALL [9]. While in Jordan the frequency of this fusion gene was 12.4% and in Kuwait it was 7% [11,12]. This difference may be related to the technique used in the studies where the latter two studies depend on cytogenetic analysis.

In relation to gender, the frequency of fusion gene was much higher in male than female, table (1), this result agreed with other studies, which found higher male/female ratio, and disagreed with other [10,13].

In present study, there was no significant correlation of the fusion gene with clinical features (table 3), however other published studies had showed that, fever, hepatomegaly, splenomegaly and LAP were more common features but CNS and testicular involvement less frequent [14].

Regarding hematological parameters only WBC count show significant difference between ETV6/RUNX1 positive cases and negative cases being higher in positive fusion gene. Other parameters including Hb, platelet bone marrow blast percent, and LDH level had no significant correlation with the presence of fusion gene. These results disagree with other studies showing that positive fusion gene cases do not have high WBC [14].

Therefore, we may conclude that ETV6/RUNX1 fusion gene is independent prognostic factor not related to other hematological and clinical parameters.

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Author contribution

Dr. Mahdi collected the cases data, performed the blood preparations, FISH procedure, statistical analyses reviewing the manuscript, Dr. Hameed and Dr. Al-Mamoori have role in study design and concept, work supervision, editing and reviewing the manuscript. Mahmood and Dr. Qassim provide technical support for FISH procedure, digital imaging using fluorescent microscope, involved in study design and reviewing the manuscript.

Conflict of interest

No conflict of interest.

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The Possible Role of HCMV in Inflammatory Bowel Diseases in Sample of Iraqi patients

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Abstract

Background Human cytomegalovirus (HCMV) reactivation is one of the most risks that occur in immunosuppressed patients. The association and role of CMV and inflammatory bowel disease (IBD) exacerbation is still controversy.

Objective To investigate the rate of occurrence and role of HCMV in patients with IBD including demographic and clinical features.

Methods A cross sectional study involved sixty-five (65) IBD patients whom divided into 9 Crohn's disease patients and 56 ulcerative colitis patients. The detection of local CMV reactivation (colon) was based on the presence of early and immediately early antigens (nonstructural proteins). The positive results for HCMV reactivation was considered according on Immunohistochemistry (IHC) and/or serological enzyme linked immunosorbent assay (ELISA) results.

Results Among the 65 eligible IBD patients, nine patients (13.85%) gave positive result for IHC, compared to 56 patients (86.15%) with negative result. On the other hand, only two patients (3.08%) had a positive result for anti-HCMV IgM antibody, while almost all patients (except one) were positive for anti-HCMV IgG antibody. There was a significant difference between positive HCMV and patients with long duration of IBD and non-response to treatment.

Conclusion IBD and its treatment may put those patients at risk of HCMV reactivation. Colonic active CMV was detected particularly in sever UC patients and significantly in patients with disease duration above 5 years and not response to treatment based on IHC technique for IE and E HCMV proteins detection.

Keywords Cytomegalovirus, inflammatory bowel disease, ulcerative colitis, Crohn's disease, immunohistochemistry

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List of abbreviations: CDAI = Crohn's Disease Activity Index, CID = Cytomegalic inclusion disease, CMI = Cell mediated immunity, HCMV = Human Cytomegalovirus, HIV = Human Immunodeficiency virus, E = Early, H&E = Hematoxylin and eosin, IE = Immediate early, IHC = Immunohistochemistry, PCR = Polymerase chain reaction, pp65 = Phosphoprotein 65, TRL = Terminal repeat long, UL = Unique long, US = Unique short

Introduction

Inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn's disease (CD), consist of chronic relapsing and nonspecific inflammatory diseases of unknown etiology that affect the digestive tract [(1)]. Multimodal approach of immunosuppressive treatment

(immunomodulators and biological agents) that used to minimize symptoms and prevent complications have suppressed the immunity in these patients which increase their risk of opportunistic infections [2-4]. Human cytomegalovirus (HCMV) is considered as one of the most common viral gastrointestinal pathogens in IBD patients [5]. Cytomegalovirus (CMV) belongs to the Herpesviridae family and represents as a common viral infection in humans, with infection level ranging from 40% in the developed countries to 100% in developing countries [6]. After primary infection, this virus is known to maintaining a persistent, long-life infection of the host, often as a latent form that can be found in different cell types [7].

HCMV infection is of particular interest in IBD that combine inflammation in the colon and the long-term maintenance of immunosuppressive therapy; both of which can reactivate latent CMV [8]. In the immunosuppressed patient with IBD, the clinical symptoms can mimic an acute exacerbation, and it is important to differentiate CMV colitis from an IBD flare-up because untreated CMV infection in these patients can lead to fulminant colitis, requiring colectomy or resulting in death [10].

Several studies have established an association between severe steroid-refractory IBD and CMV infection [11,12]; however, international guidelines from both the American College of Gastroenterology (ACG) and The European Crohn's and Colitis Organization (ECCO), which recommend as the CMV colitis should be excluded in patients with acute steroid resistance before increasing treatment dosage [13,14].

HCMV infection can be detected in both serum and tissue according to detection method. However, serum anti-CMV IgG antibodies have high specificity and sensitivity for latent infection, and IgM antibodies for acute infection or reactivation of CMV infection with viremia, but this does not correlate with active CMV colitis [15].

ECCO recommended tissue polymerase chain reaction (PCR) or histopathology combined with immunohistochemistry (IHC), using monoclonal antibodies against CMV immediate early antigen) are highly specific and sensitive for diagnosing CMV colitis in IBD [13].

There are several studies about the incidence of CMV in different parts of Iraq, Al-Obaidi in 2008 [16] studied about 32 patients with colorectal adenocarcinoma and 8 with colorectal hyperplastic polyps. Normal tissues of tumor margin were considered as control. IHC staining technique used to detect HCMV early antigens in a tissue by using specific monoclonal antibodies. CMV early Ag was detected in 5 (15.6%) out of 32 colorectal adenocarcinoma, while the other 8 patients with colorectal hyperplastic polyps and control were negative for the virus. Another study was reported by Shamran et al. in 2015 [17] was detect CMV Ags in glioma patients by using different monoclonal antibodies against different virus Ags, this study showed about 33 (91.67%), 28 (77.78%) and 26 (72.22%) out of 36 glioma samples were positive for EI-72, pp65 and late antigen respectively. In the recent study by Al-Toban et al. in 2018 [18] a sixty-one patients with acute leukemia. Forty-eight of them evaluated while induction chemotherapy (group I), while 13 post allogeneic stem cell transplantation patients, and 30 apparently healthy individuals as (control group). In this study, real-time PCR used to detect and quantitatively CMV DNA and about 12 (25%) out of 48 patients in group I, two (15.4%) out of the 13 patients in group II, and 2 (6.7%) out of 30 in the control group had positive cytomegalovirus viremia.

The aims of this study were to explore the association of HCMV infection in patients with IBD and to review the correlation of CMV infection with various demographic, therapeutic and clinical features in IBD patients.

Methods

Patients

This cross-sectional study involved sixty-five (65) inflammatory bowel disease patients whom divided into 9 CD patients and 56 UC patients. Informed consent was obtained from each patient. All patients were recruited from one hospital in Baghdad: The Gastroenterology and Hepatology Teaching Hospital/Colonoscopy Unit in the period from September, 2017 to September, 2018. Diagnosis of UC and CD was based on the presence of clinical, endoscopic, radiologic and histologic features to classify those IBD patients according to histopathologists' reports. The local Institutional Review Board (IRB) had ethically approved this study. All data were collected on patients using case note review and a questionnaire sheet that include patient demographics, age at IBD symptom onset, history, diagnosis (sign and symptoms, endoscopical and histopathological finding), medications and about other diseases. In addition to that CD activity index and partial myoscore used for CD and UC respectively, to evaluate the severity of diseases.

Samples collection

Tissue biopsies were collected during endoscopy of patients for histopathological examination to confirm all baseline data needed for this study and to obtain a tissue section slides for IHC technique. Also, blood samples were collected from each patient for serum preparation that used for detection of anti-HCMV IgM and IgG antibodies by enzyme linked immunosorbent assay (ELISA) technique.

Histopathology

Formalin fixed paraffin embedded blocks were cut into sections (5 µm thickness) used to prepare slides for Hematoxylin and Eosin staining to demonstrate typical CMV inclusions. Furthermore, two other slides were performed for IHC with two specific monoclonal Mouse Anti-Cytomegalovirus Clones (CCH2 and DDG9) to detect of early and immediate early antigens of HCMV respectively to increase the diagnostic yield of histopathology. Sections on positively charged slides were placed vertically in hot air oven at 65°C overnight. Two antigen retrieval steps done by used Trypsin as enzymatic and high pH solution (Dako) for heat protocol. A diluted primary antibody (1:50) was placed onto the tissue section and incubated for 60 minutes at room temperature in humid chamber, followed by the appropriate detection kit Dako anti–mouse HRP). Sections were analyzed via conventional light microscopy. The immunostained slides were evaluated for the presence of nuclear staining for the HCMV early antigen, sometimes accompanied by cytoplasmic staining.

Serology

An indirect ELISA were used to detect anti-CMV IgM and IgG antibodies in all patients' serum by using a commercially available kit (Forsight, USA). The positive and negative controls provided with the kit.

Statistical analysis

The statistical analysis of this study performed with (SPSS) 20.0 and Microsoft Excel 2010. Categorical data formulated as count and percentage. Chi-square test was used to describe the association between positive CMV with demographic and clinical data. Alternatively, kappa test was used to describe the agreement between diagnostic tests. The lower level of accepted statistically significant difference is below 0.05.

Results

Sixty-five patients diagnosed with active IBD (56 with UC and 9 with CD) were enrolled in this cross-sectional study. At the time of assessment, the mean age was 40.74±13.47 (range: 14-69) years. Male patients represented 46.15% of the patients (30 out of 65) and Smokers represented only a small minority of patients (5/65, 7.69%). The clinical characteristics of patients with or without HCMV are shown in Table 1. The patients were different in duration of diseases, severity of disease (mild, moderate, sever), type and response to treatment and disease extension.

CMV infection with IBD patients

Nine (13.85%) of the 65 IBD patients had CMV infection (all of them with UC) however; there is no significant difference between two types of IBD. CMV Ag was detected by IHC in tissue sample while in sera all those patients exhibited a positive Anti-CMV IgG Ab and only 2 out of 9 patients were detected with Anti-CMV IgM Ab.

Table 1. Clinical characteristics of the study population

Variable		Frequency	Percentage
Type of Disease	UC	56	86.15%
	CD	9	13.85%
Degree of Disease	Severe	29	44.62%
	Moderate	22	33.85%
	Mild	14	21.54%
Duration of disease (year)	<3	28	43.08%
	3-5	16	24.62%
	>5	21	32.31%
Response to treatment	Yes	37	61.66%
	No	23	38.34%
Type of treatment	Non biological	54	83.31%
	Biological	6	9.23%
	No treatment	5	7.69%
Disease extension	Left side colon	9	13.85%
	Proctitis	33	50.77%
	Pancolitis	23	35.38%

UC: Ulcerative colitis, CD: Crohn's disease

Possible risk factors associated with CMV reactivation

There was no significant difference in terms of age, sex, and smoking between CMV-positive and negative IBD patients ($p > 0.05$). Although, all 9 CMV-positive patients were among UC patients there was no significant difference between CMV infection and two types of IBDs. Severity of disease had shown no significant association with CMV infection although about seven CMV-positive patients out of 9 with severe illness. Only 60 patients had received treatment and about 7 (30.44%) of CMV-positive among non-response compared with only one patient among responsive was shown a highly significant differences ($p = 0.002$) with positive CMV patients. Of the 9 CMV-positive patients, seven were receiving Non-biological treatment; one was received biological treatment and one without treatment with no significant difference ($p > 0.05$). There was no significant difference in the frequency of CMV infection with respect to the disease extension of IBD ($p > 0.05$) although about 6 of CMV infection patients had proctitis involvement among 2 had pancolitis and one with left side colon. Six of the 9 CMV-positive patients had a long disease duration above 5 years shown a significant association ($p = 0.042$) than that in CMV negative patients. Risk factors for CMV infection with IBD are listed in table 2.

Table 2. Risk factors for HCMV infection with IBD

Variable		CMV		P-value
		Negative (%)	Positive (%)	
Disease	Ulcerative Colitis	47 (83.9)	9 (16.1)	0.195
	Crohn's disease	9 (100)	0 (0.0)	
Disease severity	Mild	13 (92.86)	1 (7.14)	0.095*
	Moderate	21 (95.45)	1 (4.55)	
	Severe	22 (75.86)	7 (24.14)	
Disease duration (years)	<3	27 (96.43)	1 (3.57)	0.042*
	3-5	14 (87.5)	2 (12.5)	
	>5	15 (71.43)	6 (28.57)	
Response to treatment	No	16 (69.56)	7 (30.44)	0.002**
	Yes	36 (97.29)	1 (2.71)	

*P < 0.05, ** P < 0.01

Discussion

The first published case report of HCMV associated with UC has been in 1961 lead to raise the question of whether the CMV detected was the primary cause of the patient's deterioration or a by-product of ''Ulcerative colitis, debility and the therapeutic use of adrenal cortical steroids.'' In the last 50 years this subject has become a topic in IBD literature [19].

Historically, symptomatic CMV disease was observed in immunocompromised patients; in newborns, following solid organ transplantation, in cases with human Immunodeficiency virus (HIV), or patients on immunosuppressive medications [20,21]. Numerous case series have also been reported of CMV detection in patients with severe IBD unresponsive to standard immunosuppressive therapy [22,23].

In the current study, the prevalence of HCMV in patients with IBD was 13.85%. This rate was agreement with a study in 2009 by Maher and Nassar in KSA, which detected HCMV in 9 out of 72 (12.5%) with same method of diagnosis in active IBD patients [24], Ormeci et al. 2016 in Istanbul was detect 13 out of 85 (15.4%) of IBD patients had HCMV infection [25], and Yadegarynia et al. 2018 in Iran six out 86 (7%) patients with UC the virus was detected by qPCR for colonoscopic biopsy [26].

Among 9 positive patients of IHC results, which expressed HCMV antigens (all of them had Anti-CMV IgG antibodies), only two patients had Anti-CMV IgM this situation may be due to immunosuppression status sometimes may not show IgM response as well as lower of sensitivity comparison to IHC [27,28]. Similar finding by Roblin et al. in 2011 was reported 16 patients with CMV colitis, all had serum anti-CMV IgG antibodies but none had anti-CMV IgM antibodies, although three had CMV DNA in their blood [29]. In addition, Iida et al. in 2013 found none of the 79 patients they reported with moderate or severe UC, who were anti CMV IgG antibody positive, had serum IgM antibodies to CMV [30]. Also, Gauss et al. (2015) 10-year retrospective cohort study for 294 patients with exacerbated IBD reported one patient with highly positive CMV pp65 was in the blood however CMV IgM test gave negative result [31].

According to type and severity of disease many studies have linked CMV with severe UC with prevalence ranged from 16-34% when used various diagnosis methods [12,23,30,32,33]. In case-control study performed on 226 IBD patients (83.6%), Yi et al. (2013) showed that CMV reactivation was significantly associated with severe UC patients [34]. Although, in this study there is no significant statistical difference all positive HCMV E and IE antigens IHC detected only in UC patients and about (77.7%) of severe

disease, these finding may be due to different cytokines profile of CD and UC: in CD Th 1 and Th 17 CD4+ cells differentiation with massive antiviral cytokines (IFN-γ). While there is a limited secretion of these cytokines in UC [35]. Different finding by Ormeci et al. in 2016 was reported Thirteen (15.4%) of the 85 IBD patients had CMV infection (5/42 with CD and 8/43 with UC) with no significantly different between two types of IBD [25].

Several studies and meta-analysis including 11 studies with 867 IBD patients have established an association between severe steroid-refractory IBD and CMV infection [11,12,36]. Roblin et al. in 2011 in a prospective study for 42 patients with moderate to severe UC on IV steroid treatment showed an association between CMV detection in inflamed area with resistance to steroid [29]. In this study, the association found with highly significant statistical difference (p=0.002) among seven non-response UC patients with positive for HCMV (87.5%) out from 8 positive patients takes a treatment. This association is still unclear and may be due to viral mechanism which has a role in worsening the inflammation [37].

In this study, the disease duration by years was classified to intervals (<3, 3-5, >5) according to other previous studies [31,38]. Patients with long disease duration above 5 years showed higher proportion of HCMV positive patients (28.57%) than either those with less than 3 years duration (12.5%) or those with 3-5 years duration (3.57%) with a significant difference (p=0.014), the explanation of this association may be due to CMV infection, reactivation of latent virus is a more probable event during attacks of intestinal inflammation and use of immunosuppression treatment for long period [39,40]. This significance also reported by recent study by Makarchuk et al. in 2017 during study a group of IBD patients for 6 years that about 35% of CMV infected patients were with long disease duration ≥ 5 years [38].

According to all evidences about CMV infection in patients with IBD, the management of CMV infection in IBD patients was based on the guidelines from both the ACG and ECCO, which recommend as follows: the CMV colitis should be excluded by tissue PCR or IHC in patients with acute steroid resistance before increasing treatment dosage. In patients with severe steroid resistance with detection of colonic CMV the antiviral therapy should be initiated with discontinuation of immunomodulatory agents until improve of colitis symptoms, While immunomodulatory therapy must be discontinued during systemic CMV disease [13,14].

The major findings of this study are as follows: (a) Colonic HCMV reactivation (HCMV Colitis) can occur in some IBD patients; (b) HCMV appears to have a significant role in a subgroup of IBD patients particularly refractory patients with long disease duration (>5 years) than other IBD patients; (c) The patients with severe refractory, proctosigmoiditis and older >30 year appeared to be more susceptible to HCMV reactivation; (d) The use of HCMV IE and E proteins IHC reflects the reliable method to diagnose colonic local HCMV reactivation rather than depended on H&E or serology; (e) There is a high seroprevalence of HCMV among Iraqi Patients.

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Author contribution

Fadhil: Collection of specimens, slides preparation, H&E with IHC staining and doing ELISA, preparing the manuscript and references. Dr. Kadhim: supervised the work, edit and finalize the manuscript. Dr. Al-Akayshee: Consultant Gastro and Hepatology helped in selection and providing of samples. Dr. Mirza: Consultant pathologist help in providing the histopathology reports and IHC staining results.

Conflict of interest

Authors declare no conflict of interest.

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Prevalence of Prediabetes Among Adults in Baghdad/Iraq

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Abstract

Background In prediabetes, neither individuals having diabetic range nor normal glycemic parameters in terms of fasting plasma glucose, impaired glucose tolerance or glycated hemoglobin. Two-thirds of those with prediabetes will ends eventually with type 2 diabetes. Early detection with the proper intervention will halt or reverse this progression. Data about prediabetes prevalence in Iraq are scarce.

Objective To estimate the prevalence of prediabetes among adults in Baghdad/Iraq and to identify socio-demographic and associated risk factors among the studied population and to evaluate glycated hemoglobin in the detection of prediabetes.

Methods This cross-sectional study enrolled adults (20-79 years) attending primary health care centers in Baghdad/Iraq for one year, those with known diabetes or on anti-diabetic drugs, pregnant women and those with other medical conditions that interfere with glycated hemoglobin level were excluded from the study. Data collected through direct interview. Anthropometric measurements and laboratory analysis after overnight fast were done to measure fasting plasma glucose, glycated hemoglobin and lipid profile.

Results Prediabetes prevalence was 20.6%. Prevalence was higher in older people (40-60 years) and individuals with overweight, obesity, and dyslipidemia, the agreement between fasting plasma glucose and glycated hemoglobin was very good.

Conclusion Prevalence of prediabetes in Iraq is higher than estimated and share the same risk factors to those with type 2 diabetes. Glycated hemoglobin compared to fasting plasma glucose, is a reliable test to screen for prediabetes in Iraq.

Keywords Prediabetes; intermediate hyperglycemia; glycated hemoglobin; Iraq

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List of abbreviations: ADA = American Diabetes Association, BMI = Body mass index, FPG = Fasting plasma glucose, A1C = Glycated hemoglobin, HDL-c = High-density lipoprotein cholesterol, IDF = International Diabetes Federation, LDL-c = Low-density lipoprotein cholesterol, OGTT = Oral glucose tolerance test, PHC = Primary health care, TGS = Serum triglycerides, T2DM = type 2 diabetes mellitus, WHO= World health organization

Introduction

Type 2 diabetes (T2DM) is now pandemic and is expected to persist so. In 2017, about 424.9 million (8.8%) individuals in the world (20-79 years) have diabetes and are estimated to be about 628.6 million (9.9%) in 2045, half of those are unaware of their disease especially in low and middle-income countries (84.5%). Diabetes estimated to kill about four million people in 2017 and (10.7%) of global all-cause mortality among people in this age group [(1)].

In Iraq, studies suggested a prevalence of diabetes to be (13.9%) [(2)].

At the time diagnosed, many patients with T2DM have already organ damage or advanced subclinical atherosclerosis [(3-5)]. Metabolic

abnormalities precede the onset of overt diabetes by years and are linked to decrease insulin sensitivity or increased insulin resistance. Those people share the same risk factors associated with overt diabetes (advanced age, overweight, excess calorie intake, lack of physical activity and smoking ...etc) [6]. This state in which people's oral glucose tolerance test (OGTT) or fasting plasma glucose (FPG) or glycated hemoglobin (A1C) between normal and diabetic range are defined as prediabetes. The majority of those with prediabetes will develop in future T2DM with annual conversion rate about (5-10%) and approximately 25% of them will be diabetics within 3-5 years [7,8]. Besides; people with prediabetes are at higher risk of developing many of the diabetes complications, such as diabetic retinopathy, nephropathy, neuropathy, and macro-vascular complications even before a diagnosis of diabetes has been established and thus subjected to higher healthcare expenditure [9,10].

The onset of prediabetes or the progression to T2DM can be significantly reduced or reversed through early recognition, diagnosis and proper lifestyle modifications [11]. Pooled results of 16 randomized controlled trials showed that prediabetes individuals who received lifestyle intervention had a lower rate of conversion to T2DM after one and three years of following up [12].

Worldwide, the prevalence of prediabetes is about 7.3% (4.8-11.9%) of adults (20-79) years and the vast majority (72.3%) of these individuals live in low and middle-income countries. By 2045, the prevalence expected to be 8.3% (5.6-13.9%) in this age group [1].

Iraq categorized by the International Diabetes Federation (IDF) 2017 in the Middle East and North Africa Region (MENA) and due to a lack of data, sources and information about the real situation, the prevalence of prediabetes was estimated by IDF using extrapolated data from similar ethnicity countries, geography, language, and income level [1].

In 1997, the American Diabetes Association (ADA), recommended that FPG becomes the main diagnostic test for diabetes, rather than the expensive and time-consuming OGTT [13]. In 2009, ADA recommended that the diagnosis and screening for prediabetes could also be made using A1C [14]. It is worth to mention that world health organization (WHO) does not recommend using A1C to screen prediabetes till now [15].

Although FPG was historically linked to the screening and diagnosis of prediabetes and T2DM, systematic reviews on A1C for adults (more than 40,000) adopted from 16 studies showed consistent linear association with future development of T2DM, the five-year risk for developing diabetes when A1C ≥5.7% was 9-25%, and up to 50% when A1C ≥6.0–6.5% [16]. Also, prediabetes, whether defined by A1C or FPG, is associated with a higher risk of developing T2DM [17,18].

This study aimed to estimate the prevalence of prediabetes among adults in Baghdad/Iraq and to identify socio-demographic and associated risk factors among the studied population and to evaluate glycated hemoglobin in the detection of prediabetes.

Methods

Baghdad is the capital of Iraq (5169 km^2) with a population of about 8 million. Tigris River is sandwiched by the city two halves; Karkh and Rusafa. To calculate the sample size, we assumed that 8% of the adult population would have prediabetes based on IDF prediabetes estimation in Iraq 2017, and to achieve this sample size at the 95% confidence level with an acceptable error of 5%, a single proportion formula used [19]:

$N= Z^2 p (1-p)/d2$

The selection of PHC centers had done using a multistage random sampling technique from health directorates at both sides of Baghdad yielding five health sectors sampled from Al-karkh health directorate (from a total of ten sectors) and four health sectors sampled from Al-Rusafa health directorate (from a total of nine sectors). Then four Primary health care

(PHC) centers were randomly selected from each health sector with an average ten individuals from each one, resulting in 342 adults; (178) individuals from Al-karkh Health Directorate and (164) individuals from Al-Rusafa health directorate. Diabetics or those on anti-diabetic drugs, pregnant women, those with hemoglobinopathies, malignant disease, hypo-hyperthyroidism, drugs or alcohol abuse were not included. A direct interview with each participant had done. Requested information regarding demographic data (age, sex, residence, occupation, etc.), history of smoking, hypertension, diabetes, and other medical conditions were reported.

The weight was measured (to nearest 0.5 kg), in erect position without shoes and with light clothing using an electronic scale (recommended to be used in nutrition clinics). Height was measured by using a height tape measure, which is suitable to measure a person's height with an approximation of ±0.1 cm. Body mass index (BMI) was used as an indicator of body fat, overweight, and obesity. It was calculated as body weight/height² (Kg/m²). WHO criteria were used to classify people into under, normal, overweight and obese [20].

Blood pressure was measured in a participant's arm using a mercury sphygmomanometer in a sitting position. Two blood pressure readings were taken at 5 minutes interval, and the mean value was taken. Blood pressure is expressed in millimeters of mercury (mmHg) [21]. Hypertension was considered when the systolic blood pressure equal to or above 140 mmHg and/or diastolic blood pressure equal to or above 90 mmHg or on antihypertensive drugs [22].

Laboratory analysis

A venous blood sample was obtained from each participant after confirmation of overnight fast, one-milliliter collected in a vacuum collection K3 EDTA tube (mixed thoroughly) and one-milliliter in a gel and clot activator glass tube, both stored in ice-cool box (2-8 °C) and analyzed by laboratory technician (within 4-5 hours).

Siemens Dimension EXL 200 used to measure serum FPG concentrations and the lipid profile. Venous blood sample used for A1C measurement was analyzed using the enzymatic method [ion exchange high performance liquid chromatography (HPLC) technology to separate glycated (labile A1C (L-A1c) and stable A1C (S-A1c)) and non-glycated (HbA0) forms of hemoglobin] with Arkray ADAMS A1C HA-8180V (Menarini).

Prediabetes was defined as not having previous diabetes, but having A1C between 5.7% and 6.4%, or FPG between 100 and 125 mg/dl according to ADA classification. Diabetes is considered when the FPG was 126 mg/dl or more, A1C was 6.5 or more [23].

Low density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald formula [24]:

LDL-c = [total cholesterol – (HDL-c) – [(TGS)/5].

Total cholesterol was considered high when it was ≥ 200 mg/dl. TGS high if it was 150 mg/dl or more. LDL-c was high if ≥160 mg/dl while HDL-c considered low when < 40mg/dl [25].

Data analysis

Data were coded, entered and analyzed using (Statistical Packages for Social Sciences program, version 24). Descriptive data were expressed as means and standard deviations for continuous measurements and as frequencies and percentages for categorical measurements.

Student t-test and 1-way analysis of variance were used to compare Continuous data, Chi-square test or Fisher exact test was used to test the association of Categorical data and to test agreement between testing results.

Statistical significance was accepted for a 2-sided $p < 0.05$

Results

Of the total individuals (342) enrolled, 12 (3.5%) found to be in diabetes range either by FPG or A1C and were excluded from the analysis.

Among study participants 262 (79.4%) were normoglycemic, and 68 (20.6%) had prediabetes (Table 1). Of those with

prediabetes, A1C identified 65 (95.6%) and FPG identified 55 (80.9%) individuals. Those who had prediabetes with both A1C and FPG were 52 (15.8%).

The mean age of participants was (43.8±14.4 years), those with prediabetes were older (51.5% of them between the age of 40 and 59 years) with slight male excess. The majority of them were married with lower employment and education rate. Compared to those with normoglycemia, prediabetes individuals had a higher rate of hypertension with significantly higher BMI, total cholesterol, TGS, and LDL-c. There was no significant difference between study groups concerning smoking and HDL-c level.

Table 1. Baseline characteristics for the study populations

Parameter	Total (n=330)	Normal (n=262)	Prediabetes (n=68)	P-value
Age* (years)	43.8 (14.4)	42.1 (14.6)	50.2 (11.4)	<0.001
Male sex**%	144 (43.6)	108 (41.2)	36 (52.9)	0.082
Married** (%)	262 (79.4)	202 (77.1)	60 (88.2)	0.043
Employed** (%)	144 (43.6)	116 (44.3)	28 (41.2)	0.154***
Education** (%)				
– Illiterate	45 (13.6)	27 (10.3)	18 (26.5)	0.002
– High level	89 (27.0)	76 (29.0)	13 (19.1)	
Hypertension** (%)	88 (26.7)	63 (24.0)	25 (36.8)	0.035
Current smoker** (%)	86 (26.1)	66 (25.2)	20 (29.4)	0.508
BMI*	26.8 (4.1)	26.4 (4.0)	28 (4.3)	<0.001
FPG*	91 (12)	87 (8)	108 (12)	<0.001
A1C*	5.1 (0.6)	4.9 (0.5)	6.0 (0.3)	<0.001
TC*	189 (38)	183 (34)	218 (38)	<0.001
TGS*	146 (53)	141 (47)	168 (66)	<0.001
LDL*	108 (39)	102 (35)	133 (42)	<0.001
HDL*	52 (7)	52 (7)	51 (7)	0.69

*Values are expressed as mean ± Sd

**Values are expressed as absolute number (percentage of group)

*** Fisher exact test

Statistically significant (P<0.001) agreement (kappa=0.84) was found between the results of A1C and FPG, the sensitivity and specificity of A1C was 95.3 % and 94.5%, respectively (Table 2).

Table 2. Test of agreement (FPG and A1C)

		FPG	
		Normal	Prediabetes
A1C	Normal	262	3
	Prediabetes	13	52

Kappa= 0.84, sensitivity= 95.3%, specificity=94.5%, P<0.001

Discussion

A higher prevalence of prediabetes in Iraq than that estimated by IDF may be due to the scarcity of studies regarding this subject in Iraq and thus underestimation of the real prevalence. In addition, IDF estimation relied

on measurement of impaired glucose tolerance (IGT) only as a screening tool for prediabetes, and not on other glycemic parameters (i.e. FPG or A1C) [1], considering more than one parameter in the screening for prediabetes and T2DM will boost the results [26].

Our prevalence rate was in the middle of what found in neighbored countries. For example, prevalence were 7.8% in Jordan, 11.4% in Iran and 13.8% in Qatar [27-29]. While it was higher in turkey (30.8%), Oman (44.2%) and Kuwait (44.2%) [30-32]. In Iraq, our prevalence rate was lower than that found by Al-Azzawi in Baghdad 2015 (33.7%) and what Mansour et al. found in Basrah (29.1%) [33,34]. This extreme variation reveals the complexity of the subject in term of screening tools and methods and even the sampling of the population, however, almost all studies showed an association of prediabetes with T2DM risk factors whatever the rate is.

Compared to normal participants, prediabetics were significantly older (50.4 vs. 42.1 years, $p < 0.001$), this finding is in agreement with other studies in the region [27,28]. T2DM especially attacks the elderly in developed countries while in Arab countries, it is dominated in those younger than 60 years. In Iraq, several articles were documented this fact [33-35]. In our study, more than half of those with prediabetes aged between 40 and 60 years, and this cause real impact on both economic production and health expenditure.

We found no significant difference in sex of individuals with prediabetes and this goes with the work in other parts of the world, which showed no difference or slight male excess [36,37].

Prediabetes was significantly associated with higher BMI. Also, there was a statistically significant difference in the weight of prediabetics compared to normal individuals and this goes with other studies conducted throughout the world. National Center for Health Statistics (NHANES III) estimated that 78.5% of diabetics were overweight and 45.7% were obese. A ten publications meta-analysis shows an odds ratio of 2.14 for obese subjects developing T2DM. Obesity is a strong predictor of T2DM in both genders and all ethnic groups [38,39].

The Centers for Disease Control and Prevention (CDC) in 2017 diabetes report card showed the inverse fit of diabetes prevalence with the level of education, this was consistent with our results.

Those with prediabetes had significantly higher hypertension rates with elevated lipids level except for HDL-c (Figure 1). Hypertension and dyslipidemia are well-known risk factors for T2DM [40]. The finding of very good agreement in prediabetes prevalence between FPG and A1C was incompatible with other studies, for example, the Canadian Health Measures Survey and survey of African ancestry Caribbean population [41,42]. This may be attributed to the difference in epidemiology and socio-demographic characteristics of our sample.

Our study had points of strength and limitations; it throws a light on the rising global interest in prediabetes state especially in the contest of extreme scarcity of studies in this part of the world. Besides, settings that interfere with the A1C measurement level had been restricted as much as possible. We focused on the most important epidemiological risk factors (in individuals with normoglycemia and prediabetes) that believed to play a major role in accelerating the conversion from normal to prediabetes and then to eventual T2DM, notably, the majority of them were modifiable. We tested both (A1C and FPG) in the screening for prediabetes to assess the reliability and validity of A1C alone or in combination with FPG. Recently in Iraq, A1C was approximately available at a nearly affordable cost. Our sample size enlarged before we started the study, above the minimal requirement calculated by the single proportion formula. That was because we believed that IDF underestimates the prevalence of prediabetes in Iraq and hence we enrolled more participants to augment statistical power. Also, the vast majority of our sample included individuals in PHC centers setting but they were not coming to seek medical help (e.g. Mothers accompany children for immunization, relatives of patients, some adults working

there, people coming to complete paperwork, …etc.) and thus our results can be generalized. However, of the limitations, it is an observational study and according to Bristol, "the full answers cannot be collected by observation alone" [43]. Also, the collection of past medical conditions and conditions that interfere with A1C measurement were relied on history taken from the participants and not confirmed by laboratory tests. In addition, while we used both FPG and A1C to screen for prediabetes, we didn't perform the IGT to go in line with WHO recommendations.

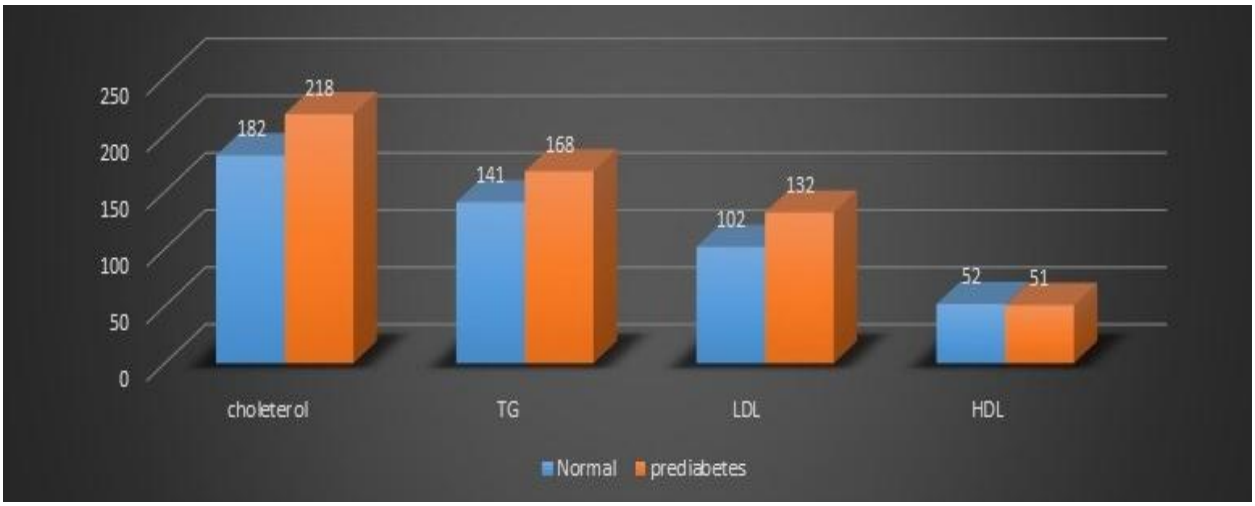

Figure 1. Lipid profile in normal and prediabetes (mean)

This study concluded that prevalence of prediabetes in Iraq is higher than estimated and necessitate more epidemiological studies to address the importance of this metabolic state. Peoples with prediabetes and T2DM were nearly similar in terms of risk factors, and hence efforts should be taken immediately to reverse this critical situation. A1C, compared to FPG, is a reliable test to screen for prediabetes in Iraq. More and larger studies are needed to assess the epidemiology of the condition and to further evaluate prediabetes screening modalities in Iraq.

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Author contribution

Dr. Alogaili: collected data, analyzed them and prepared the manuscript. Dr. Alsaffar: study design and manuscript revision. Dr. Hamid: final revision of the manuscript.

Conflict of interest

The authors declare no conflict of interest for the present study.

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Efficacy of Laparoscopy in The Management of Unilateral Nonpalpable Testis

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Abstract

Background Undescended testis is one of the most common malformations seen in the field of pediatric surgery. The most problematic aspect of undescended testis is the diagnosis and treatment of nonpalpable testis. Laparoscopy has been widely used for the diagnosis and treatment of nonpalpable testis.

Objective To evaluate the role of laparoscopy in the diagnosis and treatment of unilateral nonpalpable undescended testis.

Methods This is a prospective study carried out in the period from December 2012 to December 2017 in the Pediatric Surgery Department of a tertiary hospital in Baghdad. We used laparoscopy in the diagnosis and treatment of 40 patients aged between one and 12 years (median age 4.9 years) with unilateral nonpalpable undescended testis. Boys with a palpable testis at any point were excluded from the study. Surgical procedure was individualized according to the laparoscopic findings either by one stage laparoscopic orchiopexy, two stage Fowler-Stephens procedure or laparoscopic orchiectomy.

Results Laparoscopy was able to diagnose the site of the nonpalpable testes in all the patients. Out of 40 nonpalpable undescended testes, 26 testes (65%) were intra-abdominal (12 testes were low intra-abdominal, 14 testes were high intra-abdominal). In 9 patients, (22.5 %), the vas deferens and spermatic vessels were found entering the internal inguinal ring. In 3 patients, (7.5 %), the testes were vanishing, and the testes were absent in 2 patients (5%). All patients with low intra-abdominal testes (n=12) were subjected to one stage laparoscopic orchiopexy through the normal inguinal ring. Out of 14 patients with high intra-abdominal testes, 7 patients underwent two staged Fowler-Stephens laparoscopic procedures, while three patients were treated by laparoscopic Prentiss maneuver and the remaining 4 patients underwent immediate laparoscopic orchiectomy due to presence of an atrophied testis. Patients with the vas deferens and spermatic vessels entering the internal inguinal ring (n=9) were treated by orchiopexy via conventional inguinal approach.

Conclusion Laparoscopy for unilateral nonpalpable testis has an excellent diagnostic yield combined with high success rate following repair.

Keywords Laparoscopy, nonpalpable undescended testis, Fowler-Stephens procedure

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List of abbreviations: None

Introduction

Undescended testis or cryptorchism is the most common genital problem in male children [(1)]. It occurs in

approximately 3% of term male infants and in up to 33-45% of premature infants [2]. However, the prevalence of cryptorchidism drops to 1% at end of 12 months [3]. Cryptorchism is associated with a variety of potential consequences like neoplasia, infertility, testicular torsion, inguinal hernia, psychological stigma, and parents' anxiety. Treatment of undescended testes is aimed at minimizing these risks [4,5]. The clearest classification divides testes into palpable (80%) and nonpalpable (20%). The nonpalpable testes may be due to intra-abdominal location, vanishing testis, agenesis, inguinal location with a different grade of dysplasia or atrophy, or ectopic testis [6].

The mainstay of therapy for the palpable undescended testis is orchiopexy with creation of a subdartos pouch. For a unilateral unilateral undescended testis that is not palpable under anesthesia, initial management may be either through diagnostic laparoscopy or inguinal exploration. In the last decade, laparoscopy has become the preferred approach [7]. It has replaced ultrasound and magnetic resonance imaging for the localization of a nonpalpable testes and become the most widely used and most useful diagnostic modality in the management of the nonpalpable testis [8]. Laparoscopy is the most sensitive and specific procedure to localize the nonpalpable testis with an accuracy rate of over 95% [9]. The primary advantage of laparoscopy over initial inguinal exploration for a nonpalpable testis is that laparoscopy avoids injury to the collateral vasculature that may occur with initial inguinal dissection [10].

Options for dealing with the intraabdominal undescended testis include:

1. If the testicular vessels appear blind ending, some have recommended no further exploration, although this is controversial.
2. If the testicular vessels are seen entering the internal ring, inguinal exploration is performed [7].
3. If the testicular vessels end blindly in the inguinal canal, the tip of the vessels can be sent for pathologic examination.
4. If diagnostic laparoscopy reveals a viable intra-abdominal testis, several options are available depending on its location, including:

A. If the gonadal vessels are long enough and the testis lies caudal to the iliac vessels, orchiopexy may be performed via open or laparoscopic approach depending on surgeon preference [11].

B. When the gonadal vessels are too short, there are various options:

i. A neoinguinal ring may be created medial to the inferior epigastric vessels to shorten the path for scrotalization of the testis (Prentiss maneuver) [7].

ii. A staged orchiopexy can also be performed in which the high abdominal testis with its cord structures is first mobilized as low as possible. Six to 12 months later, it is mobilized into the scrotum.

iii. Two-stage Shehata orchiopexy can be performed laparoscopically. In the first stage the intraabdominal testicleis first mobilized and then the gonad is placed on tension within the abdomen. In the second stage, further mobilization of the testicle into the scrotum is performed while preserving the spermatic vessels [12].

iv. Alternately, a two-stage Fowler-Stephens orchiopexy can be performed typically laparoscopically. In the first stage the tethered testicular artery is divided. In the second stage, after 6 months when collaterals have formed, the testis is brought down on a wide pedicle of peritoneum containing the remaining vessels [13].

v. The single-stage Fowler-Stephens procedure can also be performed [13].

vi. Other options include microvascular orchiopexy (autotransplantation).

5. If the testis is atrophied, whether found in the abdomen or the inguinal canal, a laparoscopic or open orchiectomy is recommended. Debate exists regarding the role of contralateral fixation in cases of monarchism because of differing assumptions related to potential torsion. This largely remains the surgeon's preference [7].

This study aimed to evaluate the role of laparoscopy in diagnosis and treatment of unilateral undescended testis.

Methods

This is a prospective study of 40 patients with unilateral nonpalpable undescended testis conducted over the period from December 2012 to December 2017 in the Pediatric Surgical Department in a tertiary hospital in Baghdad. The study included all patients aged between one and 12 years with unilateral nonpalpable undescended testis whereas patients with palpable undescended testis at any point and those with bilateral nonpalpable undescended testes were excluded from the study.

A special data form had been used including variables such as name, age, clinical examination, investigations (including ultrasonography and magnetic resonance imaging), anatomical site affected, laparoscopic finding and follow-up findings after 6 months-2 years.

After confirming the diagnosis, a written informed consent was taken from each patient's parent or guardian. All patients were examined under general anesthesia with muscle relaxation and endotracheal ventilation, the inguinal region and scrotum of the affected side were carefully palpated.

Laparoscopy was performed with the patient in supine position. Small umbilical incision was made and a 5 mm umbilical port was inserted. Thereafter, the peritoneal cavity was insufflated with CO_2 under a pressure of 6 to 10 mmHg. After the insertion of the telescope, hollow viscera and other organs were assessed to exclude injury. Next, the internal inguinal ring, vas deferens and spermatic vessels, testicular size and position were evaluated. Comparison with the contralateral side was made. Then two (5 mm) working ports were inserted at both iliac fossae for orchiopexy and vessels clipping and transaction. Subsequent surgical procedure was individualized according to the laparoscopic findings.

1. Intraabdominal testes:
   a. Low intraabdominal testes (<2.5 cm from deep inguinal ring): single-stage laparoscopic orchiopexy through normal deep inguinal ring
   b. High intraabdominal Testis (≥2.5 cm from deep inguinal ring): two-stage Fowler–Stephens procedure or Prentiss maneuver.
2. When vas deferens and vessels were found entering the ring: inguinal exploration with assisted laparoscopy followed by orchiopexy.
3. Vanishing testes (blind ending vessels) and absent testes (no vas and vessel): no intervention was required
4. Atrophied testes: Laparoscopic orchiectomy.

All patients who underwent laparoscopic procedure were discharged from the hospital on the next day. Thereafter the patients were followed up at regular intervals (6 months to 2 years). The testes assessed by clinical examination, ultrasound and color doppler study for its position and size. For outcome analysis, success was defined as a testis that remained in the scrotum with no atrophy or decrease in size at a follow-up.

Results

Within the study period, a total of 40 patients underwent diagnostic and therapeutic laparoscopy for unilateral nonpalpable testis. The age ranged between 1 and 12 years, the median age was 3.9 years. Twenty-six patients (65%) had right sided nonpalpable testes, while 14 patients (35%) had left sided nonpalpable testes.

During laparoscopy, A total of 26 testes (65%) were detected intra-abdominal, including 12 patients (30%) with low intra-abdominal testes

within 2.5 cm of internal inguinal ring, and 14 patients (35%) with high intra-abdominal testes. The laparoscopic treatment of intra-abdominal testes varied according to their morphology and position. In patients with low intra-abdominal testes (n=12), one stage laparoscopic orchiopexy through normal inguinal ring had been performed. Seven out of 14 patients of those with high intraabdominal testes underwent two staged Fowler–Stephens laparoscopic procedure with initial vascular transection. Three patients were subjected to laparoscopic Prentiss maneuver. In the remaining four patients with high intra-abdominal testes, the testes were found to be atrophied, so immediate laparoscopic orchiectomy had been done for them (Table 1) (Figure 1).

In nine patients (22.5%) the vas deferens and spermatic vessels were seen entering the internal inguinal ring. These patients were subjected to open orchiopexy using the conventional inguinal approach. In one of them hypoplastic testis was detected. Vanishing testes were found in three patients (7.5%) while the testis was absent in two patients (5%). No surgical treatment was needed in these five patients (Tables 1 and 2), (Figure 2).

Table 1. Diagnostic findings of laparoscopy in nonpalpable undescended testis

Diagnosis	No. (%) of patients
Low intra-abdominal testis (within 2.5cm from deep inguinal ring)	12 (30%)
High intra-abdominal testis (more than 2.5 cm from deep inguinal ring)	14 (35%) (10 not atrophied and 4 atrophied)
Vas and vessels entering the inguinal ring	9 (22.5%)
Vanished testis (blind-ended vessels and vas deferens)	3 (7.5%)
Absent testis	2 (5%)
Total	40 (100%)

Table 2. Therapeutic approach for nonpalpable undescended testis

Therapeutic approach	No. (%) of patient
Single stage laparoscopic orchiopexy through normal inguinal ring	12 (30.0%)
Two stages Fowler-Stephen laparoscopic procedure	7 (17.5%)
Prentiss maneuver (single stage laparoscopic orchiopexy)	3 (7.5%)
Laparoscopic orchiectomy for atrophied intraabdominal testes	4 (10.0%)
Open orchiopexy (when the spermatic vessels are entering inguinal ring)	9 (22.5%)
No surgical intervention (vanished and absent testis)	5 (12.5%)
Total	40 (100%)

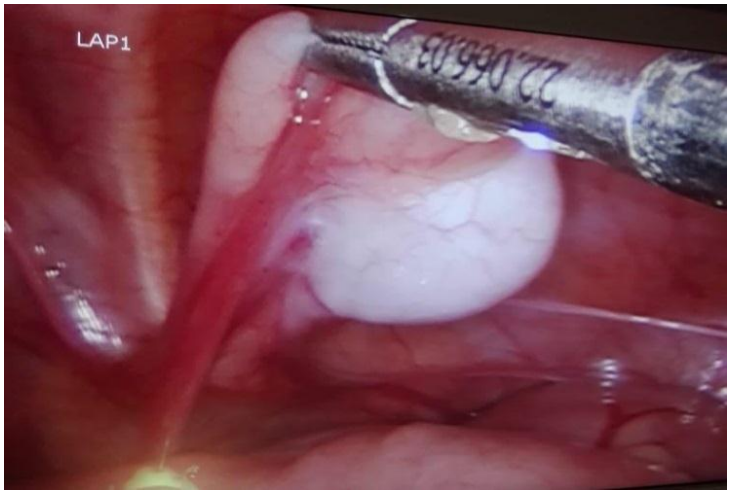

Figure 1. The testis is within 2.5 cm of deep inguinal ring

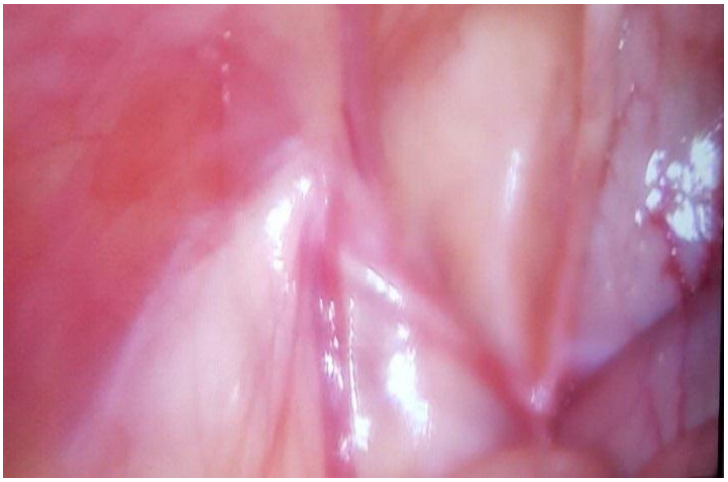

Figure 2. The testis is within 2.5 cm of deep inguinal ring

Regarding period of follow-up (6 months to 2 years with median follow up of 12 month) after operation, patients with orchiectomy and vanishing testes had been excluded from follow up (9 patients). By clinical examination, ultrasound and color doppler study, a good size and morphology of the testes were found in 28/31 patients (90.3%) whereas 3/31 (9.7%) testes had been found to be atrophied during follow-up. Two of these atrophied testes were became atrophic after 2 stages Fowler-Stephens technique, and the remaining other atrophied testicle was becoming atrophic after conventional inguinal approach for open orchiopexy (this was hypoplastic during primary orchiopexy). The successful rate of laparoscopic orchiopexy in patients with intraabdominal testes was (90.9%) (Table 3).

All testes underwent single stage laparoscopic orchiopexy were located in their hemiscrotums with good size. Whereas 5/7 (71.4%) testes which had 2 stages Fowler-Stephens laparoscopic orchiopexy were found in their hemiscrotums with good size and remaining 2/7 testes were atrophic.

Table 3. Postoperative follow up regarding site and size of testis

Site and size of the testis	No. (%) of patient
Good scrotal position and size of testis	26 (83.8%)
Good scrotal position but atrophied testis	2 (6.5%)
Testis at the neck of scrotum with good size	2 (6.5%)
Atrophied testis at the neck of scrotum	1 (3.2)
Total	31 (100%)

Discussion

An undescended testis is one of the most common clinical disorders of childhood [14]. About 20% of undescended testes are nonpalpable on physical examination [15]. Nowadays, laparoscopy is the most reliable diagnostic modality in the management of nonpalpable testes [16].

In our series, laparoscopy was used as a tool for diagnosis and definitive management of unilateral nonpalpable testes in 40 patients over a period of 5 years (2012-2017). The median age group of our study was 3.9 years with patients from 1 to 12 years. Zubair et al. [17] have reported similar median age group as 4 years (9 month-12 years). The mean age of presentation reported by Zouari et al. [18] was 3.8 years. Despite the recommendations for the treatment of the undescended testis before 2 years of age, many of our patients were older. Illiteracy, ignorance and poor awareness, late referral to the surgical clinic in and low socioeconomic condition may be the reason for this late presentation in our patients. Tang et al. [16] identified that the main cause of delay in presentation to the surgical clinic was due to late referral of patients.

Regarding the laterality of nonpalpable undescended testes we found that undescended testes is more common on the right side than the left side, which is similar to Hamidi et al. [19] study who reported right sided undescended testes in 61% and left sided in 39% of all patients. The commonest position of nonpalpable testes in our study was intra-abdominal 26 out of 40 (65%) of which 30% were low intra-abdominal and 35% were high intra-abdominal). Other studies found that the percentage of intra-abdominal testes range from 52% to 87% [20-23]. In our study morphology of the testis was correlated with the position of testis and the actual age of the patient which revealed that features of atrophy were higher in high intra-abdominal testis and older age groups and this was similar to that reported in Humphrey et al. [24] and Boeckmann et al. [25] studies.

During the follow-up period, all 12 testes that underwent single stage laparoscopic orchiopexy were located in their hemiscrotums with good size, which translate to a success rate of 100%. Whereas 5 out of 7 testes, which had two staged Fowler-Stephens laparoscopic orchiopexy were found in their hemiscrotums with good size while 2/7 were found to be atrophied. So, the success rate of 2 stages Fowler-Stephens laparoscopic orchiopexy in our study was 71.4%. Most of the unsuccessful outcomes involved the high intra-abdominal testis with very short pedicle. Other studies reported similar success rates for single and two staged laparoscopic orchiopexy [17,26]. The two-stage laparoscopic Fowler-Stephens procedure is currently the most popular technique for intra-abdominal testes, with success rate of about 80–85% [27,28].

During the study period, laparoscopic orchidopexy for intra-abdominal testes provided an overall success rate of (90.9%). The success rate of operation was varies from 74- 91.1% in the literature [29].

In this study, the deep inguinal ring with vas deferens and vessels traversing it were found in 9 patients (22.5%). All these patients underwent conventional inguinal exploration with orchiopexy. The significance of this fact was that these patients had testis in the superficial inguinal pouch. The difficulty in

palpating the testis could be contributed to obesity, the small size of the testis or peeping testes. Other studies described a percentage of inguinal testes in range of 24-42% [22,23,26,30].

In this study, the testes were vanishing in three patients (7.5%) and absent in two patients (5%) due to agenesis. In these patients, laparoscopy has benefit in avoiding unnecessary groin exploration. Zubair et al. [31] and Godbole et al. [32] have reported that unnecessary exploration can be avoided in 20% and 42% cases, respectively. Denes et al. [30] reported that laparoscopic surgery was the definitive diagnostic method in patients with testicular agenesis or vanishing testis and saved these patients from any further incision or unnecessary investigation.

Collectively, the diagnostic yield of laparoscopy in our study was 100% and the overall therapeutic yield was 87.5%, as there were five patients (12.5%) with vanishing and absent testes on laparoscopy. Dar et al. [22] has reported 100% diagnostic yield of laparoscopy and 96.9% therapeutic yield as they could localize and manage 32 nonpalpable testes with only one vanishing testes on laparoscopy.

This study concluded that laparoscopy for unilateral nonpalpable testis has an excellent diagnostic yield combined with high success rate following repair, which agree with previous studies.

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Author contribution

Dr. Zain and Dr. Fadil: collection of data, statistical analysis and writing the first draft of manuscript. Dr. Mohammed and Dr. Abdul-Hassan made the final draft of manuscript.

Conflict of interest

The authors declare no conflict of interest in publishing this article on competitive intention.

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Effect of Topical Flavonoid Fraction from *Artemisia annua* in Comparison with Tacrolimus on Induced Atopic Dermatitis in Mice

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Abstract

Background Atopic dermatitis is a chronic inflammation of skin disease that is characterized by recurrent acute pruritus and dry skin. Mostly, atopic dermatitis is predominant in young children. The problems of increasing prevalence and high impact of disease on quality of patients and family's life, necessities identifying many atopic dermatitis prevention planes.

Objective To determine the effect of *Artemisia annua* flavonoids fraction in comparison with tacrolimus in atopic dermatitis like mice model.

Methods This study is a prospective, randomized, placebo and controlled animal designed. Thirty-two male Albino mice that six weeks age included in this study. The mice were randomly divided into four groups. Group I without treatment (Healthy). Group II only inducer, phthalic anhydride used. Groups II, III, and IV subjected to phthalic anhydride solution, which was applied on the dorsum of the back skin at 9 A.M. three times a week for four weeks. After three hours of phthalic anhydride application, treatment is used for group III (Tacrolimus 0.03% ointment), and group IV (flavonoids fraction 1.2 mg /kg ointment) topically once daily at 12 P.M. for three times a week for four weeks). Serum IgE and immunohistochemistry of skin tissue IL-4 score, and IL-13 score were measured.

Results High significant decrease in immunohistochemistry of skin tissue IL-4, and IL-13 in flavonoid fraction group were found.

Conclusion The flavonoid fraction has an effect on the skin immunohistochemistry parameters and probably on atopic dermatitis like mice model.

Keywords Atopic dermatitis, *Artemisia annua*, flavonoids

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List of abbreviations: A.M. = Before noon, AD = Atopic dermatitis, Ig = Immunoglobulin, IHC = Immunohistochemistry, IL = Interleukin, P.M. = After noon, Th = T helper, FLG = Filaggrin, TSLP = Thymic stromal lymphopoietin

Introduction

Atopic dermatitis (AD) is a chronic inflammation of skin disease that is characterized by recurrent acute pruritus, eczematous rash and dry skin. Mostly, AD is predominant in young children especially in those children with a genetic tendency to atopic march diseases [(1)].

The problems of increasing prevalence and high impact of disease on quality of patients and family's life, necessities identifying many AD preventions planes. The avoidance of allergy is not beneficial with no emerging of regular approach [(2)].

There is an evidence of psychological and physical trouble associated with AD. AD is characterized by erythema, skin xerosis, lichenification, and exudative topical damage. The most difficult presentation to control is pruritus which contributes effectively to disease worry. AD has been found to increase the probability of depression and anxiety and effect life quality [3,4].

A probable effect of different ratios of genetic and non-inherited environment factors may be found. Early age onset AD in patients expected more liable to genetic effect, while in young babyhood and adult onset AD patients, the progress of the illness may be associated with more causes of environmental factors. In spite of that, few associations between filaggrin (FLG) mutations loss of function and the onset of AD in studies of early babyhood have been identified [5].

The pathophysiology of AD is complex and involves genetic problems, immune abnormalities, skin barrier damage, the microbiome, and environmental triggers. Increased expression of the cytokine related to T helper (Th) 2 pathway occurs in AD, with IL (Interleukin)-4 and IL-13 are the major players in this disease [6]. The Th2 cytokines production can start activation of eosinophils, eosinophil inflow, and deposition of eosinophil substances, such as major basic protein in skin lesion [7]. In addition to that, cytokines of Th2 upregulate high affinity receptors of immunoglobulin (Ig) E on antigen presenting cells, example Langerhans cells, and more encourage synthesis of IgE antibodies. IgE-attached Langerhans cells in the existence of activated keratinocytes that secret IL-25, TSLP, and IL-33, are highly effective crossing to regional lymph nodes then presenting the allergenic substance to naïve T cells and start a Th2 response [8].

Treatment topically with glucocorticoids or calcineurin inhibitors is the main therapy for the management of AD, and the use of systemic anti-inflammatory use of glucocorticosteroids for short term, cyclosporine use in adults and azathioprine used in some severe cases of AD [9]. However, problems with corticosteroid in long term use can cause side effects such as weak immune system, dependency, and skin thinning with darkening [10].

Therefore, a safe and effective original AD treatment therapy is needed to establish better outcome with a least side effect. *Artemisia annua* used because of its various chemistry and biology effect of the constituents, and the national source of the plant material in Iraq. In the present study probable useful therapeutic effects of *Artemisia annua* flavonoids fraction, will be evaluated in AD as well as investigating possible difference in serum IgE, immunohistochemistry (IHC) of IL-4, and IL-13 in a mice model of AD and healthy groups.

Methods

This study is a prospective, randomized, placebo and controlled animal designed. The study was done in the Department of Pharmacology in College of Medicine, Al-Nahrain University. Thirty-two male Albino mice that are six weeks age included in this study. The protocols for the animal experiment used in this study were carefully reviewed for ethical and scientific care procedures and approved by Institutional Review Board (IRB); Approval date 4/2/2018.

The mice were randomly divided into four groups (each group eight). Group I without treatment (Healthy). Group II only inducer, Phthalic Anhydride (Prepared by dissolving phthalic anhydride in 4:1 of freshly mixed aceton and olive oil) [11] given. Groups II, III, and IV subjected to 100 microliters of 5% phthalic anhydride solution which was applied on the dorsum of the back skin at 9 A.M. three times a week for four weeks to induce a state of that resemble atopic dermatitis. After three hours of phthalic anhydride application, treatment is used for group III (Tacrolimus 0.03% ointment) [12], and group IV (flavonoids fraction 1.2mg /kg ointment) topically once daily at 12 P.M. for three times a week for four weeks). Flavonoids

fraction dose is calculated according to fraction representation percent in the plant [13].

The plant *Artemisia annua* is collected from north of Iraq, dried and saved in AL Jadria Herbal Store according to the document from University of Baghdad, College of Science, Department of Biology Approval Number 8 in 12-4-2017. Five hundred grams of shad dried *Artemisia annua* leaves coarse powder were macerated in hexane for 24 hours and then dried at room temperature. The defatted plant materials were extracted with ethanol 80% in soxhlet apparatus. The ethanolic extract is evaporated using rotary evaporator at temperature not exceeding 40 ^{o}C. This Crude fraction was acidified with the addition of hydrochloric acid (5%) to reach pH 2 and then equal volume of ethyl acetate is added to get two separated layers. The ethyl acetate layer was evaporated to dryness using rotary evaporator under reduced pressure and then basified with 300ml of sodium Hydroxide 5% to reach pH 10 and extracted with chloroform in the separator funnel to get two separated layers. The aqueous basic layer was separated, evaporated to dryness and then acidified with hydrochloric acid 5% to reach pH 2 and finally extracted with ethyl acetate to get flavonoids fraction [14].

Immunoglobulin E measured quantitatively by the enzyme-linked immunosorbent assay (ELISA) (Using mice serum IgE kit, catalog number: CSB-E07983m, Cusabio-China). After incubating the tested serum in an antigen-coated polystyrene plat or tube, enzyme specifically labeled anti-immunoglobulin is then added and this enzyme then remaining in the plate or tube after washing gives a measure to the quantity of specifically related antibody in the serum [15].

IHC study is done (Using IHC kit of IL-4 and IL-13 catalog number: Orb318722 and Orb10895 respectively, Biorbyt-USA) to determine IL-4 and IL-13 that present in the skin tissue lesion of mice, an IHC technique was initially standardized at the IHC Laboratory of the Department of Microbiology with the aid of consultation center in Department of Pathology, College of Medicine, Al-Nahrain University. The fundamental principle is the demonstration of antigens inside tissue sections by method of use specific antibodies. The immunoglobulin target molecule has special binding sites for each antigen and for other antibodies. Antigen-antibody attachment binding is measured with a colored histochemical change visible by fluorescent or light microscopy [16].

Statistical analysis was done by analyzing data using computer facilities of Statistical Package for Social Sciences (SPSS) version 25 and tests of mean, standard deviation, and independent t-test were done.

Results

It was found a high significant increase (P value ≤ 0.001) in serum IgE, IHC of IL-4, and IL-13 in AD induced non-treated group when compared to healthy group. Table (1), Figure (1), and Figure (2). When AD induced non-treated group compared with Tacrolimus group, a high significant decrease in serum IgE, IHC of IL-4, and IL-13 was found (Table 2). While when compared with flavonoid fraction group, a significant decrease in serum IgE and a high significant decrease in IHC of IL-4, and IL-13 (Table 3).

Table 1. Comparison between healthy group and atopic dermatitis induced non-treated group

Parameter	Healthy mean±SD	Atopic dermatitis mean±SD	p value
Serum IgE level	2.26±3.06	22.88±13.95	<0.001**
IHC IL-4 score	1.0±0.0	4.0±0.1	<0.001**
IHC IL-13 score	0.0±0.0	4.0±0.0	<0.001**

** Denote high significant difference at P value ≤ 0.001

Table 2. Comparison between atopic dermatitis induced non-treated group and tacrolimus group

Parameter	Atopic dermatitis mean±SD	Tacrolimus mean±SD	p value
Serum IgE level	22.88±13.95	2.67±4.78	0.001**
IHC IL-4 score	4.0±0.1	1.5±0.55	<0.001**
IHC IL-13 score	4.0±0.0	1.0±0.0	<0.001**

** Denote high significant difference at P value ≤ 0.001

Table 3. Comparison between atopic dermatitis induced non-treated group and flavonoid fraction group

Parameter	Atopic dermatitis mean±SD	Flavonoid fraction mean±SD	p value
Serum IgE level	22.88±13.95	4.36±6.86	0.004*
IL-4 score	4.0±0.1	2.5±0.55	<0.001**
IL-13 score	4.0±0.0	3.0±0.0	<0.001**

* Denote significant difference at P value ≤ 0.05

** Denote high significant difference at P value ≤ 0.001

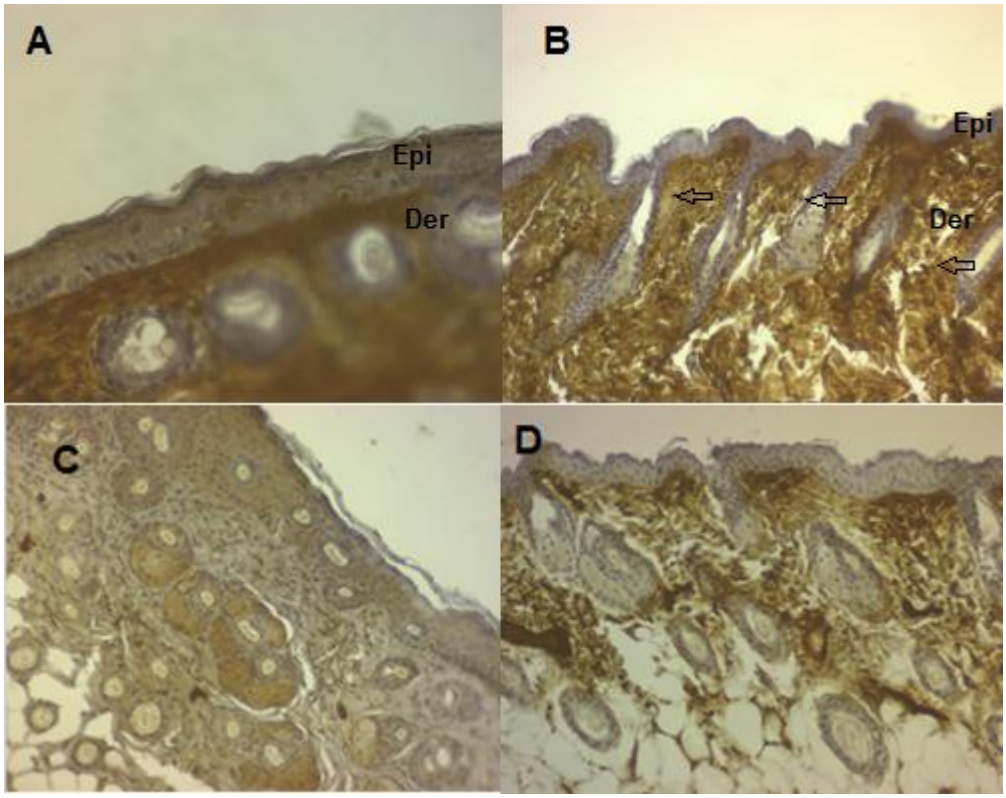

Figure 1. Immunohistochemistry of IL-4 score of healthy group (A) (40x) and Atopic dermatitis Induced non-treated group (B) (20x), Tacrolimus group (C)(20x), and Flavonoid fraction group (D) (20x) showing IL-4 positive reactions (Arrow indicates dark brown)

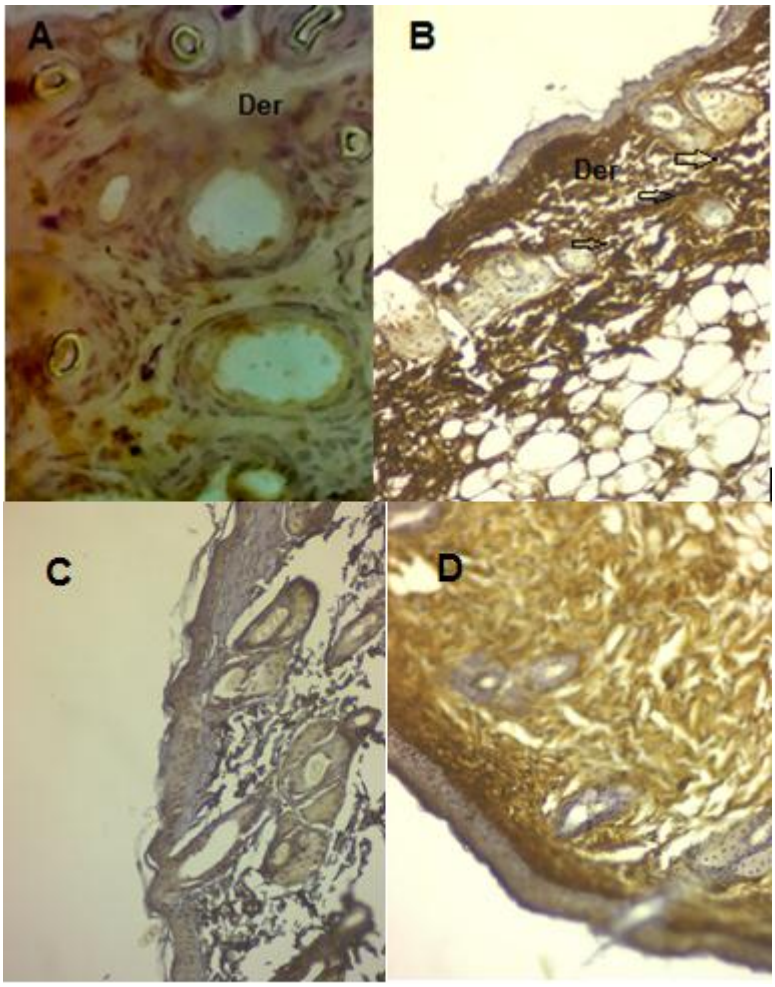

Figure 2. Immunohistochemistry of IL-13 score of healthy group (A) (40x), Atopic dermatitis Induced non-treated group (B) (20x), Tacrolimus group (C)(20x), and flavonoid fraction group (D)(20x) showing IL-13 positive reactions (Arrow indicates dark brown)

Discussion

High significant increase in serum IgE was recorded in AD group when compared to healthy group. This result was comparable with a study showed that repeated skin application of phthalic anhydride solution lead to a significant increase in serum IgE levels in the induced non-treated group [17], and it is clearly also show that the total IgE was higher in pediatric AD patients [18]. High significant increase were shown in IHC of IL-4 in the skin of AD group, which is the same result stated that the skin lesions show significant increased levels of IL-4 in mice model skin lesion of AD [19]. In the epidermis of transgenic mouse with overexpressing IL-4, the animal develop all the specific symptoms of AD including pruritus, skin bacterial infection, increased skin inflammatory cells, and high IgE and IgG1 [20]. High significant increase was shown in IHC of IL-13 in the skin tissue of the mice of AD group. This is identical with the study that noted high significant elevation in IL-13 in the stratum corium in atopic dermatitis group when compared with healthy normal group [21].

High significant decrease in serum IgE was found in this study in tacrolimus treatment group, which is similar to the result of a study that showed tacrolimus significantly suppressed the increased serum IgE concentration [22]. Tacrolimus cause immunosuppression through decreasing

responses of T lymphocytes to foreign allergic antigens in addition to suppressing IL-2 cytokine transcription, which is the main pathway. It controls transcription of several genes that code for many inflammatory mediators like IL-2, tissue necrosis factor-alpha, granulocyte-macrophage colony-stimulating factor, interferon-gamma as well as other interleukins, which are required for immune responses development. Tacrolimus also suppress histamine release from mast cells [(23)]. It was found that high significant decrease in tissue IL-4 and in IL-13 in tacrolimus treated group when compared with AD induced non-treated group. Through signal transducer, the cytokines affect functions of epidermal barrier and transcription 6 activator. As an example, IL-4 and IL-13 decrease the expression of filaggrin, involucrin, loricrin, and desmoglein 3 in keratinocytes [(24)]. Moreover, IL-4 and IL-13 increase the function and expression of a kallikrein 7, chymotrypsin serine protease in epidermal keratinocytes. This leads to high protease activity, and finally epidermal barrier dysfunction [(25)].

It has been found that after 4 weeks of treatment with topical flavonoid fraction, significant decrease in serum IgE when compared with AD induced non-treated group. The anti-inflammatory effect of flavonoid fraction may be due to its suppression of mitogen activated protein kinase signaling pathway and nuclear factor-kappa B [(26)]. Finally, it was found that high significant decrease in IHC of IL-4, and IL-13 in flavonoid fraction group. It is not sure obviously that flavonoids anti-inflammatory effects due to either their modulation of a single pathway. It is suspected that flavonoids hit on an attached network of transcription factors and kinases that can coordinately exert a defend response to the pathological stress that exposed by chronic inflammation [(27)].

This study concluded that the flavonoid fraction has an effect on the skin IHC scores of IL-4 and IL-13 in mice and probably is an option in the treatment of atopic dermatitis in the future.

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Author contribution

Hameed: collection and analysis of data, interpretation and discussion. Dr. Abu-Raghif: research reviewer and Dr. Kadhim: identification plant extract procedure.

Conflict of interest

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Review Article;

Nanoparticles Technology in Medicine, As A Diagnostic Tool, and Therapeutic Applications for Many Chronic and Genetic Diseases

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Abstract

Nanoparticle is an artificial cell-like particle (antigen-presenting artificial cell that can be tuned to target a specific disease or infections). The outer surface of each particle is covered with universal adaptor molecules having attachment points for antigens, specific molecules on specific cells, and fight off the targeted disease.
Inside of each particle, there is either cytokines, cytotoxic drugs, antimicrobial drugs, genetic material, iron, gold, herbs, and others; each for different curative purpose, yet all of them act locally with high specificity to avoid devastating side effects of the contents if given systemically or to target certain tissue for curing diseases due to genetic deletions, or as a vaccine. Different nanoparticles differ in size, shape, contents, material of the outer shell, and purpose (i.e. for diagnosis of cancer, fighting that cancer, dealing locally with autoimmune diseases, treating a disease with genetic deletion mutations, fighting an infection, monitoring, and control of biological systems.

Keywords Nanoparticles, diagnostic tool, therapeutic applications

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Introduction

Nanoparticles (NPs) are the most commonly used nanotechnology structures, consisting of two or more dimensions on the nanometer scale, according to the American Society for Testing and Materials (ASTM). Compared to their corresponding bulk materials, they have different enhanced chemical and physical properties, such as a high surface area-to-volume ratio and a specific quantum size effect due to their unique electronic structures [(1)]. The properties of NPs, in addition to their composition, depend on their size and shape [(2)]. To reduce aggregation and obtain monodispersed NPs, it is necessary to control their size and shape by facilitating their cell internalization [(3)].

Types of nanoparticles

NPs are classified into three main groups according to their chemical compounds: organic nanoparticles (liposomes, polymers), nonorganic nanoparticles (metals, metal oxides, ceramics, and quantity dots), and carbon-based nanoparticles [(4)]; different types' shapes shown in figure (1).

Liposome Nanoparticles

These are spherical vesicles containing an aqueous material with an outer lipid bilayer. The materials used to prepare these vesicles are amphiphilic, close to biological membranes,

in order to improve the efficacy and safety of different drugs [5]. Liposomes are used primarily for the delivery of chemotherapeutic drugs in cancer treatment [6].

Polymeric Nanoparticles

Most are considered to be biodegradable and biocompatible, and are the most frequently used NPs in drug delivery systems [7-9]. These are either made from natural polymers like chitosan or synthetic polymers like poly-lactides (PLA), poly-methyl methacrylate (PMMA), or poly-ethylene glycol (PEG) [7]. To improve the efficiency of drug loading and prolong the release of drugs, consideration must be given to the existence of polymer-drug interactions, the form of polymer and its physical-chemical properties [10].

Metallic Nanoparticles

They are either valuable metals (gold, silver) or magnetic metals (doped ferrites of iron oxide, cobalt and manganese). Metallic nanoparticles such as gold (Au) have unique electronic and optical characteristics and are non-toxic and biocompatible with other biomolecules due to their negative charges [11-12]. A surface of gold has the ability to conjugate ligands such as proteins, oligo nucleotides, and antibodies with functional groups such as phosphins, thiols, mercaptans, and amines [13]. Gold nano-conjugates coupled with strongly enhanced localized surfaces (gold plasmon resonance nanoparticles) can be used for the treatment of various diseases in imaging techniques [14].

Metal Oxide Nanoparticles

They have catalytic, antioxidant, chemical, optical, and biocompatibility activities that make them suitable for many biomedical applications. The most widely used types are ironoxide (Fe_3O_4), Titania (TiO_2), Zirconia (ZrO_2), and later Ceria (CeO_2) [15]. Titania nanoparticles, like a biosensor [16], are assembled into restorative inserts and used in critical applications. Ceria nanoparticles are able to switch between oxidation states, especially cerium (IV) and cerium (III) oxidation states, due to the proximity of multiple surrenders on their surface, enhancing their application in oxidation-related stress-related diseases [17]. Porous silica (SiO_2) has unique properties, including large surface area, pore volume, controllable particle-size, and good bio-compatibility make them very useful in the delivery of drugs [18].

Ceramic Nanoparticles

These are non-organic compounds used as drug carriers with porous properties. They can carry molecules like proteins, enzymes, or drugs without compromising swelling or porosity due to pH or temperature effects [19]. Silica and aluminum are the most commonly used materials of ceramic nanoparticles. But it is also possible to use a mixture of metallic and non-metallic materials [20]. For example, CeO_2-capped mesoporous silica nanoparticles, "MSN," were established as carriers for the delivery of therapy by releasing β-cyclo-dextrin into lung cancer cells [21].

Most types of ceramic materials are available with multiple applications, such as clay minerals, cement, and glass. Bio-ceramics, which have good biocompatibility, hydrophilicity, osteoconductivity, biodegradability and reabsorbability, are primarily used for bone, teeth and other medical applications, Calcium phosphate (CaP), calcium sulphate and carbonate, tri-calcium phosphate (TCP), hydroxyl-apatite (HAP), TCP + HAP, bio-active glasses, bio-active glass ceramics, titanium-based ceramics, alumina ceramics, zirconia ceramics, and ceramic polymer composites are the most commonly used ceramic nano-bio-materials. In addition to other bio-medical uses in the human body, most of them were used in nano-medicine, orthopedics, bone regeneration, dentistry, and tissue development [22].

Quantum Dots

Quantum dots (QDs) are made of a semi-conductor core (such as cadmium-selenium

(CdSe), cadmium-tellurium (CdTe), indium-phosphate (InP), or indium-arsenate (InAs)), over-coated with an outer layer (such as zinc-sulfide (ZnS)) to improve optical and physical properties and to prevent leakage of toxic heavy metals [23]. To be used in bio-imaging and bio-sensing strategies, they need to be combined with biomolecules such as proteins, peptides, or oligo-nucleotides that enable them to bind to specific sites [24].

Carbon-Based Nanoparticles

They are considered of interest in biomedical applications because of their high electrical conductivity and excellent mechanical power, but they are not bio-degradable and require surface modifications as they have a strong tendency to form large aggregates [25-27]. Either they are fullerenes or nanotubes. Fullerenes are novel allotropes of carbon with a polygonal structure consisting solely of 60-carbon atoms [28]. Carbon nanotubes are generally made from the deposition of chemical vapor graphite. There are two types of carbon nanotubes: single-walled (SWCNT) and multi-walled (MWCNT), the latter with strong anti-microbial properties [29]. Carbon nanotubes (CNTs) have amazing optical properties, which is why they are used as agents for labeling and imaging [28]. In fact, CNTs have optical transitions (transition of their electrons from orbit to another lead to transmission of energy in form of light in the near infrared (NIR) region), making them useful in biological tissue and cells, since NIR has lower excitation scattering and greater depth of penetration [30].

Therefore, in the NIR field, fluorescence shows much lower auto-fluorescence than ultraviolet or visible ranges. Therefore, for NIR fluorescence microscopy and optical coherence tomography, CNTs are efficient imaging agents with higher resolution and high tissue depth. That's why Cherukuri et al. controlled CNTs successfully in phagocytic cells and mice (intravenously administered) using NIR fluorescence [31].

Medical application of nanoparticles

Generation of oxidative stress

An increase in the levels of reactive oxygen and nitrogen species (RONS) derived from physiological cellular oxidation is characterized by oxidative stress. The antioxidant system fights the excess in RONS under normal conditions in order to maintain the organism's equilibrium. The imbalance that promotes oxidative stress is usually associated with several artery dysfunction-related pathological conditions such as hypertension, atherosclerosis, diabetes mellitus, or acute coronary syndrome [32]. NADPH-oxidase (Nox) [33], uncoupled endothelial enzyme NO-synthase (eNOS) [34], xanthine-oxidase (XO) [35] and enzymes in the respiratory chain [36]. Sources of reactive oxygen species (ROS) within the vascular wall are known. Under physiological conditions, Nox overwhelms, as Nox is associated with an increase in xanthine-oxidase activity, eNOS uncoupling and mitochondrial ROS production [36]. It should be noted that angiotensin II (AT II) is associated with vascular ROS production by increasing the expression of Nox [37] and XO [38] and reducing thioredoxin (antioxidant system) [39]. Blood flow exerts a frictional force on endothelial vascular cells, namely hemodynamic shear stress, which ultimately leads to ROS release [40]. Shear stress is released by eNOS from L-arginine by endothelial nitric oxide (NO). NO is a strong vasodilator [41] that prevents platelet adhesion and aggregation, leukocyte chemotaxis [42], vascular smooth muscle proliferation [43], anti-atherogenic effects [44], and increases the growth factor of the endothelial vascular system. Also included in the vascular wall are anti-oxidant processes such as superoxide dismutase (SOD), catalase, glutathione peroxidases, thioredoxin system, and peroxideroxins.

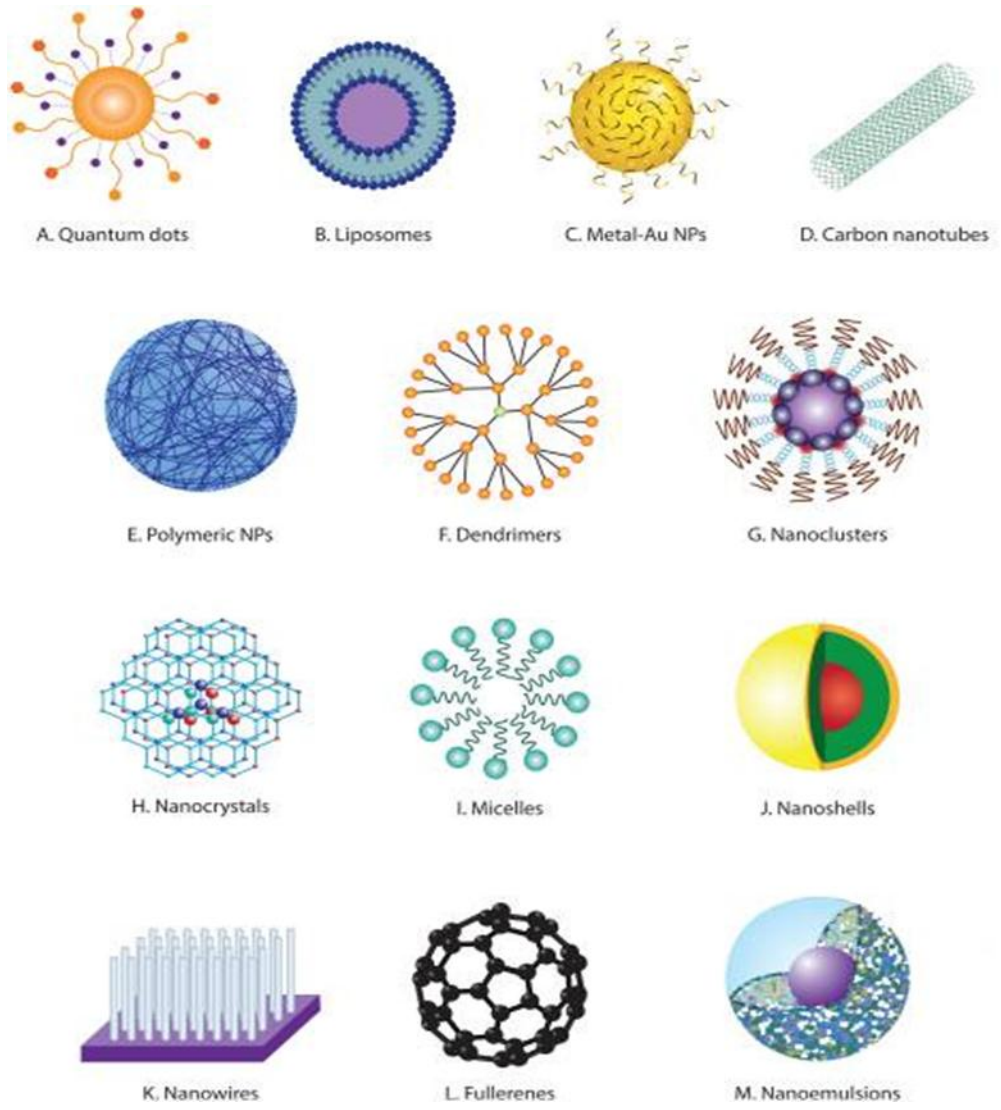

Figure 1. Shapes of different types of nanoparticle. From Bench to Bedside [45].

Nanoparticles for targeting vascular oxidative stress

Uncoupled endothelial NO (eNO) nanoparticles

An interesting recent study [46] developed a hybrid molecule consisting of a copolymer (poly lactic-co-glycolic acid) (PLGA) nanoparticle containing SA-2 and having functionalities both anti-oxidant and NO donor and supplying a sufficiently therapeutic level of NO to cure peripheral arterial disease.

Angiotensin II converting enzyme (ACE) inhibitor nanoparticle

Both Ile-Pro-Pro (IPP) or Val-Pro-Pro (VPP) are the best anti-hypertensive peptides obtained (inhibiting angiotensin-converting enzyme (ACE)). Nevertheless, due to their gastrointestinal deterioration, they are impaired by their poor oral bio-availability. The use of nanoparticles to encapsulate these peptides could therefore prevent their proteolysis and increase their systemic absorption. A study by Yu et al. [47] PLGA nanoparticles (PLGANPs) tested in a model of essential hypertensive rats as an oral delivery system for anti-hypertensive small peptides.

The final conclusion was that PLGANP was a hypertension treatment that was potentially effective.

Nanoparticle with natural anti-oxidants mimetic activity

Ceria (CeO_2) nanoparticles are likely to restore vasodilatation depending on the endothelial. Minarchick et al. studied the impact of nanoceria on vascular reactivity in hypertensive rats and concluded that the microvascular dysfunction and oxidative stress associated with hypertension were reduced by these nanoceria [(48)]. Nano-ceria contain a high O_2 vacancy density in their structure, allowing them to store O_2 during the lean process and return O_2 to metal particles during the oxygen-rich phase. This capacity is referred to as ceria's O2 storage capacity [(17)]. Several experiments have shown that nanoceria is in vitro SOD mimetic [(49)] and has antioxidant and anti-inflammatory activity in the myocardium murine [(50)]. CeO2NPs can therefore have cardiovascular-protective effects that make them endothelial inflammatory controllers. Hence, beneficial effects on oxidative stress in cardiovascular diseases can be achieved by inducing nanoparticles to overproduce H_2O_2. Poly-oxalate has been shown to have anti-oxidative and anti-inflammatory properties-producing nanoparticles, since they have been able to limit the impact of H_2O_2 on ischemia / reperfusion injury [(51)]. Researchers have developed nanoparticles carrying SOD1 that have been shown to enhance cardiac function after myocardial infarction [(52-53)]. Some nano-materials, such as nano-ceria, have mimetic multi-enzyme activities because they can imitate SOD, catalase, oxidase, phosphatase, and peroxidase. For this reason, nano-ceria can scavenge radicals of hydroxyl and nitric oxide [(54)]. In this sense, cerium nanoparticles have great potential to cure oxidative stress-related diseases, since most nano-materials only scavenge one form of RONS.

Early detection of cancer utilizing nanotechnology

Because tumor cells grow faster than the normal ones, so neovascularization occurs to fulfill their requirements for nourishment and oxygen, those new blood vessels are yet abnormal, i.e. are leaky, and lacking effective drainage as shown in figure (2). Therefore, scientists could make use of this phenomenon to settle these nanoparticles in cancerous cells, in addition to that, nanoparticles can reach cancerous cells even if they have metastasized - or spread to other organs in the body.

In the fight against cancer, half of the battle is won based on its early detection. Nanotechnology provides new molecular contrast agents and materials to enable earlier and more accurate initial diagnosis.

For cancer, nanodevices are being investigated for the capture of blood borne biomarkers, including cancer-associated proteins circulating tumor cells, circulating tumor DNA, and tumor-shed exosomes. Nano-enabled sensors are capable of high sensitivity, specificity and multiplexed measurements. Next generation devices couple capture with genetic analysis to further elucidate a patient's cancer and potential treatments and disease course.

Nanotechnology based imaging contrast agents being developed and translated today, offer the ability to specifically target and greatly enhance detection of tumor in vivo by way of conventional scanning devices, such as magnetic resonance imaging (MRI), positron Emission tomography (PET), and computed tomography (CT). Moreover, current nanoscale imaging platforms are enabling novel imaging modalities not traditional utilized for clinical cancer treatment and diagnosis, for example photoacoustic tomography (PAT), Raman spectroscopic imaging and multimodal imaging (i.e., contrast agents specific to several imaging modalities simultaneously). Nanotechnology enables all of these platforms by way of its ability to carry multiple components simultaneously (e.g., cancer cell-specific targeting agents or traditional imaging contrast

agents) and nanoscale materials that are themselves the contrast agents of which enable greatly enhanced signal [55].

Researchers at Stanford University and Memorial Sloan Kettering Cancer Center developed multimodal nanoparticles capable of delineating the margins of brain tumors both preoperatively and intra-operatively. These MRI-PAT-Raman nanoparticles are able to be used both to track tumor growth and surgical staging, by way of MRI, but also in the same particle be used during surgical resection of brain tumor to give the surgeon 'eyes' down to the single cancer cell level, increasing the potential tumor specific tissue removal [56].

For metastatic melanoma, researchers at Memorial Sloan Kettering Cancer Center (MSKCC) and Cornell University have developed silica-hybrid (SiO_2) nanoparticles ('C-dots') that deliver both PET and optical imaging contrast in the same platform. These nanoparticles are actively targeted to the cancer with fouropore, cyanine 5.5 (Cy5.5) and surrounded by polyethylene glycol (PEG) chains attached to cyclo-(Arg-Gly-Asp-Tyr) cRGDY peptides that target this specific tumor type and have already made it successfully through initial clinical trials [56].

Similarly, gold nanoparticles are being used to enhance light scattering for endoscopic techniques that can be used during colonoscopies. One really powerful potential that has always been envisioned for nanotechnology in cancer has been the potential to simultaneously image and deliver therapy in vivo and several groups have been pushing forward these 'theranostic' nanoscale platforms. One group at Emory University has been developing one of these for pancreatic cancers, which are traditionally harder to deliver therapeutics to. Their platform for pancreatic cancer can break through the fibrotic stromal tissue of which these tumors are protected by in the pancreas. After traversing through this barrier, they are composed of magnetic iron cores which allow MRI contrast for diagnosis and deliver small-molecule drugs directly to cancer cells to treat [55].

Finally, nanotechnology is enabling the visualization of molecular markers that identify specific stages and cancer cell death induced by therapy, allowing doctors to see cells and molecules undetectable through conventional imaging. A group at Stanford has developed the Target-Enabled in Situ Ligand Assembly (TESLA) nanoparticle system. This is based off nanoparticles which form directly in the body after IV-injection of molecular precursors. The precursors contain specific sequences of atoms, which can only form larger nanoparticles after being cleaved by enzymes produced by cancer cells during apoptosis (i.e., cell death) and carry various image contrast agents to monitor (PET, MRI, etc.) local tumor response to therapies. Being able to track cancer cell death in vivo and at the molecular level is extremely important for delivering effective dosing regimens and/or precisely administering novel therapies or combinations [55] as shown in figure (3).

Nanotechnology for treatment of cancer

Magnetic nanoparticles (MNPs) for treatment of cancer

These are able to convert electromagnetic energy into heat [57]. Therefore, the most popular application for MNPs is most likely the destruction of tumor cells by heating them to their apoptosis threshold [58]. A study illustrated the successful use of spin-vortex, disk-shaped permalloy magnetic particles in a low-frequency, rotating magnetic field for the in vitro and in vivo glioma destruction [59].

Nanoparticles as photosensitizing drugs treatment (PDT) for cancer

Is an externally-active and minimally invasive technique for treatment of cancer. The process of PDT involves the systemic or local use of photo-sensitizing drugs, called photo-sensitizers (PSs), then a photo-excitation of the PSs in the tissue using light of the appropriate wave-length and power [60]. In oxygen presence, the PS is excited from the ground

state to the excited state after activation with light of an appropriate wave-length, and an electron is transferred to nearby tissue oxygen, producing oxygen free radicals or excited singlet oxygen i.e. ROS [61-63], leading to cell damage, and eventually to cancer tissue damage. To enhance the effect of PSs, building a targeted drug delivery system with MNPs has become of interest. For instance, a study by Park et al. [64] synthesized multifunctional cobalt ferrite ($CoFe_2O_4$) NPs ($CoFe_2O_4$-hematoporphyrins (HPs)-FAs) functionalized by coating them with HP for introducing photo-functionality and by conjugating with FA for targeting cancer cells. Pyropheophorbide-a (PPA) as a novel chlorin PS was prepared for PDT. PPA-coated multifunctional magneto-fluorescent NPs, Fe_3O_4, SiO_2, CS, PPA (MFCSPPA) were designed. The experiments demonstrated that MFCSPPA had strong photodynamic therapy activity and low dark toxicity [65].

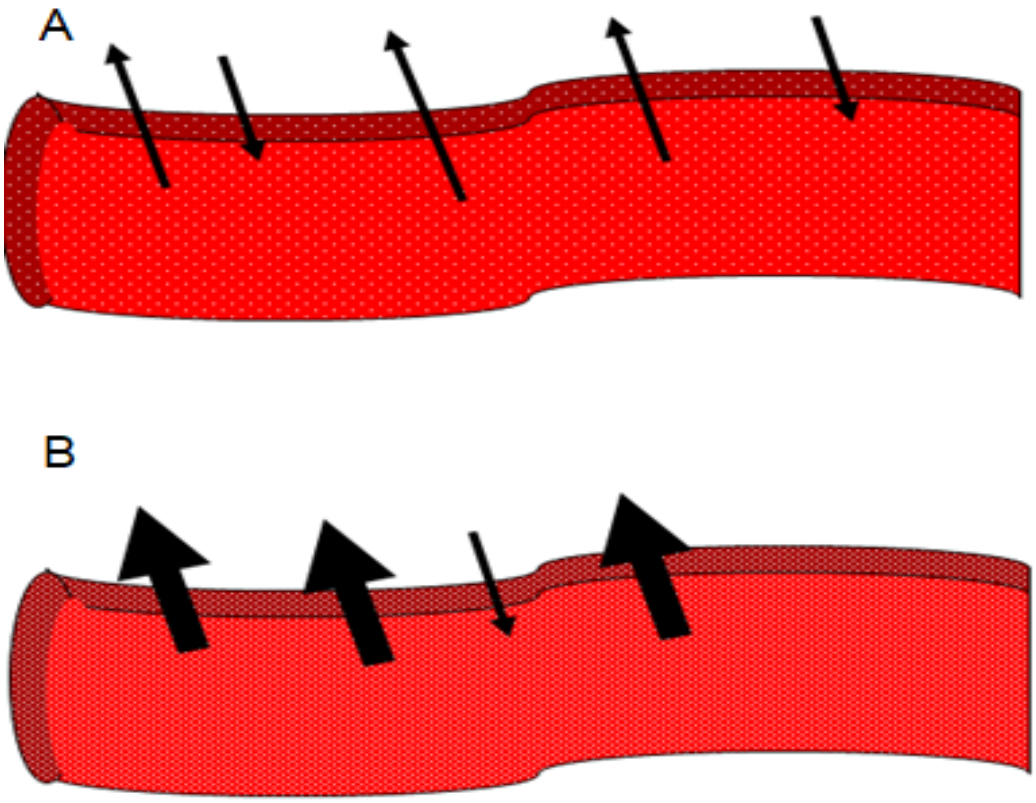

Figure 2. A) Normal blood vessels have selective capacity for passage of molecules, and with effective drainage, (thickness of the arrows is indicative to the size of molecules getting in or out of the vessel, while number of the arrows is indicative to the number of molecules getting in or out of the vessel). B) Blood vessels of cancerous tissues, don't have selective capacity for molecular passage in or out of the vessel, and are leaky, with defective draining capacity, so larger molecules can pass out of them to cancerous tissues, and reside there as they can't drain them back effectively. Credit: National Cancer Institute [54]

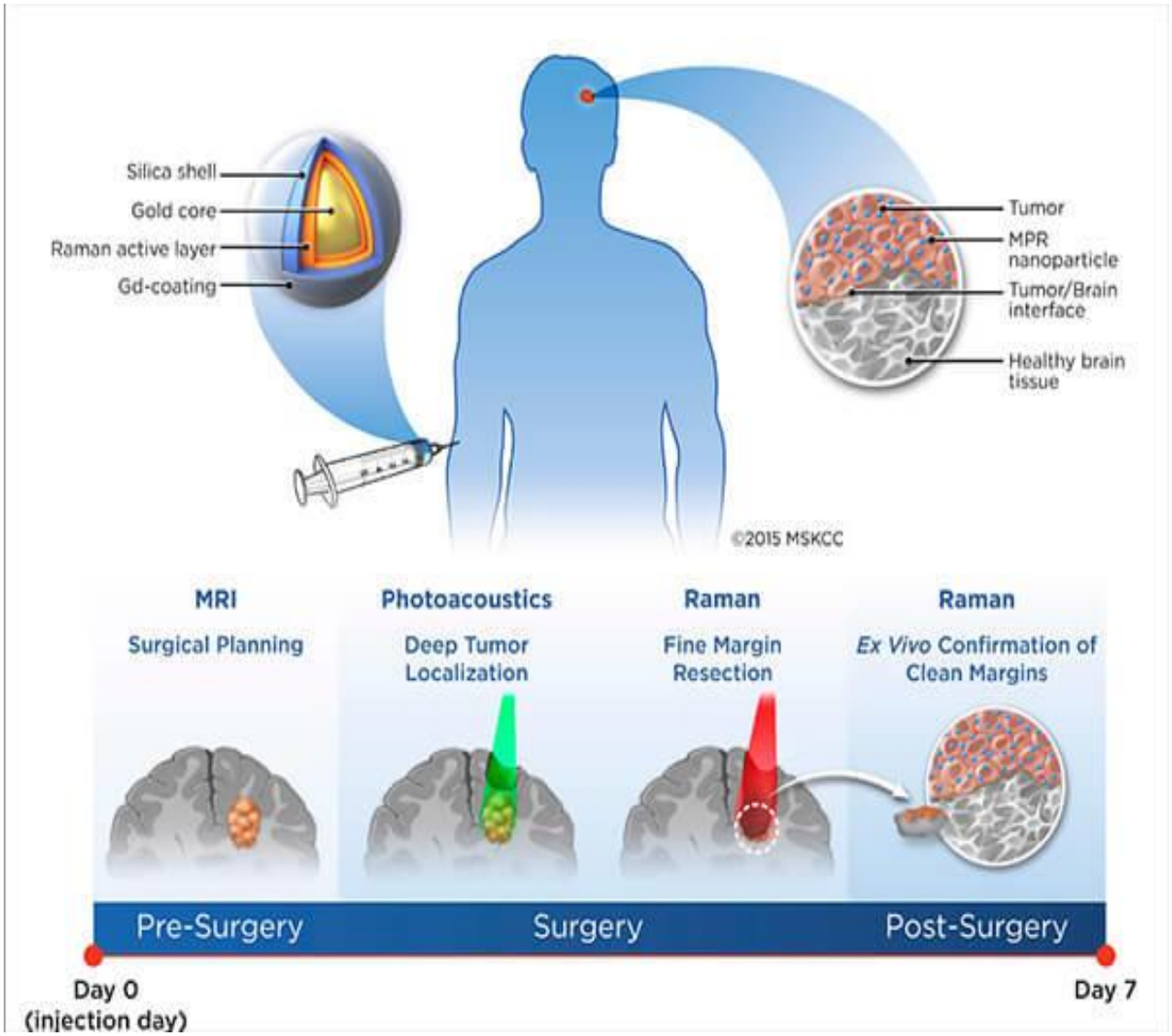

Figure 3. Principle of a triple-modality MRI-photoacoustic-Raman nanoparticle for clinical use. The nanoparticle is injected intravenously. In contrast to small molecule contrast agents that wash out of the tumor quickly, the nanoparticles are stably internalized within the brain tumor cells, allowing the whole spectrum from preoperative MRI for surgical planning to intraoperative imaging to be performed with a single injection. T1-weighted MRI depicts the outline of the tumor due to the T1-shortening effect of the gadolinium. During the surgery, photoacoustic imaging with its greater depth penetration and 3D imaging capabilities can be used to guide the gross resection steps, while Raman imaging can guide the resection of the microscopic tumor at the resection margins. Raman would be used for rapid conformation of clean margins in the operating room instead of the time-consuming analysis of frozen sections

Nanoparticle as photo-thermal treatment (PTT) for cancer

Despite that near infra-red (NIR) is with low toxicity on skin and deep tissue penetration, yet it may directly kill cancer cells by PTT, which has become a controlled treatment strategy [66]. PTT using photo-thermal agents in combination with NIR has also gained increasing attention for cancer treatment [67]. An example of this is engineering phosphopeptide-decorated MNPs as efficient photo-thermal factor for solid tumor treatment [68]. Compared with individual magnetic Fe_3O_4 NPs, clustered Fe_3O_4 NPs may result in a marked increase in NIR absorption [69]. Upon NIR irradiation at 808 nm, clustered Fe_3O_4 NPs

inducing higher temperatures were more cytotoxic against A549 cells [69]. In the majority of cases, PTT and MRI are carried out in combination [70,71]. However, a study indicated that, compared with their large counterparts, small Fe_3O_4 NPs exhibited greater cellular internalization, thus enabling a higher PTA efficacy in vitro [72]. In addition, 120 nm may be the optimal diameter of Fe_3O_4 NPs for MRI and PAT in vitro [72]. Therefore, the size of MNPs may be an important factor for PTT.

Nanoparticles as carrier for lethal therapies for cancer

Anti-cancer drugs (chemotherapy, hormone and biological therapy) are the choice for metastatic cancers that are currently used. Chemotherapy works by separating rapidly growing cells, a characteristic of cancer cells, but it also affects normal cells with rapid proliferation rates, sadly, like hair follicle cells, bone marrow and gastrointestinal tract cells, this leads to common chemotherapy side effects. Because of these side effects as well as the development of multidrug resistance, there has been a need to find new effective targeted therapies based on changes in tumor cells' molecular biology. Targeted cancer drugs approved by the Food and Drug Administration (FDA) in recent years, block biological transduction pathways and/or specific tumor proteins to induce cancer cells apoptosis in addition to immune system stimulation, or specifically deliver chemotherapeutic drugs to cancer cells, reducing unwanted side effects. Targeted therapies can be performed directly by modifying specific cell signals by using monoclonal antibodies or small molecular inhibitors to over-expressed receptors on the surface of tumor cells [73].

Nanoparticles as carriers for chemotherapeutic drugs

To deliver the anti-cancer drugs directly to bone tissue, nanoparticles were developed attracted to calcium, which concentrates in high levels in bones. This done by covering the surface of the nanoparticles with a substance known as alendronate, which binds to calcium. Then these spheres engineered to carry an anti-cancer drug called bortezomib. When these tiny particles were tested in mice with myeloma, it was found that they could find and target the cancer cells present in the bone. The treatment slowed the growth of tumors while also strengthening the bone in these mice. There by these engineered targeted therapies manipulate the tumor cells in the bone and surrounding microenvironment to effectively prevent cancer from spreading in bone [74].

Bisphosphonates (BPs) are commonly used to treat bone disease due to their high bone tissue affinity. Makes BPs useful for bone tissue delivery of NPs. BPs' traditional applications are promoting the prevention of fracture, healing, or osteoporosis, and Paget's bone disorder disease. The emerging evidence, however, indicate that BPs also have anti-tumor activity and can be used for treatment with cancer bone metastases. Preclinical studies have shown that second-generation BPs (zoledronic acid) can inhibit angiogenesis, invasion and adherence of malignant cells, and overall progression of cancer, indicating their ability to block bone metastasis growth. Serum levels of the vascular endothelial growth factor (VEGF), a critical factor for angiogenesis, have been significantly reduced in patients receiving zoledronic acid in clinical studies, indicating that zoledronic acid may be capable of inhibiting angiogenesis. BPs of the third generation (risedronate (RIS)) have recently been available and are assumed to be more effective with less toxicity [74].

Nanoparticles as carriers for lethal gene therapy for cancer treatment

1. (Rexin-G)

Rexin-G is a nanoparticle designed to deliver a fatal gene directly into tumor cells, this trial, designed to test the safety of the drug primarily, the agent was well-tolerated, without treatment-related side effects. There were only 9 patients enrolled in this study, but what the authors found interesting was that all 9 patients had either stable disease or partial response (more responses with the higher dose tested) of their tumors. Rexin-G is now being evaluated in larger phase II studies in pancreatic cancers, and sarcomas [75].

2. The "Trojan Horse" therapy

In this trial a package of RNA is delivered into cancer cells, this RNA signal is called a "silencing RNA", or siRNA. This siRNA signals a cancer cell to stop production of proteins that cause chemotherapy resistance. A second mini-cell is then injected which delivers chemotherapy drugs into the cancer cells. So far, this Trojan Horse approach has the potential to treat a large number of different types of cancer, and particularly some of those with very poor survival rates like pancreatic cancers [76].

Nanotechnology for diagnosis, treatment of autoimmune diseases

Autoimmune diseases (ADs) are chronic conditions initiated by the loss of immunological tolerance to self-antigens. The diagnosis of ADs depends on the identification of disease-associated clinical signs and symptoms as well as the detection of autoantibodies.

A. Diagnostic techniques

ADs can be organ specific e.g., type I diabetes mellitus (T1DM) or systemic (e.g., systemic lupus erythematosus (SLE)). Therefore, an important group of targets are disease-related membrane antigens. These antigens can act as biomarkers and could help define the phenotype of the disease and sometimes identify therapeutic targets.

Gold NPs, one of the NPs used in this respect, have the potential biocompatibility, relatively low short-term toxicity, high absorption coefficient and physical density compared with other metal NPs [77]. Other important NPs are iron NPs, which have been used for more than two decades as contrast agents for MRI. These particles can be organized according to their hydrodynamic diameter into several categories: standard superparamagnetic iron oxide particles (SPIOs) (50 to 180 nm), ultrasmall superparamagnetic iron oxide particles (USPIOs) (10 to 50 nm), and very small superparamagnetic iron oxide particles (VSPIOs) (< 10 nm) [78]. Tourdias et al. reported that combination of gadolinium and USPIO in patients with multiple sclerosis (MS) can help identify additional active lesions compared with the current standard, the gadolinium-only approach, even in progressive forms of MS [79].

This method uses iron-oxide NPs that are targeted to sites of complement activation with a recombinant protein that contains the C3d-binding region of complement receptor 2. Iron-oxide NPs darken (negatively enhance) images obtained by T2-weighted MRI [80]. Due to its unique ability to directly image myocardial necrosis, fibrosis and edema, cardiac magnetic resonance (CMR) is now considered the primary tool for noninvasive assessment of patients with suspected myocarditis. Moon et al. has described a CMR imaging with magneto-fluorescent NP that allows visualization of myocardial inflammation cellular infiltrates and distinction of the extent of the inflammation compared with conventional CMR in a preclinical model of experimental autoimmune myocarditis in rats [81].

Recently, Gaglia et al. [82] developed a noninvasive method to visualize T1DM at the target organ (pancreas) in patients with active insulinitis; using magnetic resonance imaging of magnetic NPs. The authors visualized islet inflammation, manifested by microvascular changes and monocyte/macrophage recruitment and activation. PET, single-photon emission computed tomography (SPECT) technologies in combination with radiolabeled immunoglobulin derived targeting probes could be used for tracking inflammatory cells in vivo. Dearling et al. [83] described the use of radio-labeling of an anti-β7 integrin antibody with the positron-emitting radionuclide ^{64}Cu in detecting acute colitis in experimental murine model with the aid of micro-PET. It was found that higher uptake of the radio-labeled antibody in the intestine of mice with acute colitis compared with controls observed by using both micro-PET imaging and ex-vivo tissue assay, suggesting that the β7 integrin monomer could be used as the target for colitis imaging, and that the radio-labeled antibody targeting a subset of lymphocytes, can serve as a specific imaging tool.

Nanobodies are the smallest antigen-binding antibody-fragments, that shows fast and

specific targeting in vivo and have low immunogenicity due to their large sequence identity with human VH genes of the VH III family [84]. Recently, Put et al. [85] reported the use of SPECT/micro-CT imaging with 99mTc-labeled Nanobodies directed against the macrophage mannose receptor for monitoring and quantifying joint inflammation in collagen-induced arthritis, a mouse model for rheumatoid arthritis (RA). The authors showed that macrophage mannose receptor is expressed on macrophages in vitro and in vivo in synovial fluid of inflamed paws, whereas expression is relatively low in other tissues.

B. Therapeutic techniques

Current therapeutic strategies against ADs may be divided into three main classes: (1) sign and symptom amelioration therapies, i.e., non-steroidal anti-inflammatory drugs (NSAIDs); (2) medications to change the normal nature of the illness, including disease-modifying anti-rheumatic drugs (DMARDs) for biological and non-biological diseases; and (3) therapies directed to the complications resulting from the disease-associated organ damage [86].

Steenblock et al. [87] mimicked physiological antigen presentation by engineering NPs, which influence the particle-phagocyte interaction as has been demonstrated by Mitragotri and colleagues [88], who invented microcapsules mimicked live mouse red blood cells. They demonstrated three preliminary examples: surface-absorbed hemoglobin for oxygen delivering, encapsulated iron oxide nanocrystals as imaging contrast agents, and encapsulated heparin as an anticoagulant. New strategies to deliver anti-inflammatory drugs to innate immune cells selectively and inflamed tissues and reverse their pathological phenotypes are of great interest as a therapeutic tool for ADs. Nano-delivery systems are capable of reducing drug dose and administration frequency by extending half-life and increasing the metabolic stability of small molecules. Nano-carriers can preferentially accumulate in arthritic joints due to enhanced vascular permeability at inflammation sites where they are subsequently phagocytozed by recruited monocytes/macrophages, to activate them and eventually inducing their apoptosis [89].

Yuan et al. [90] developed a novel pH-sensitive drug delivery system of dexamethasone (Dex) specifically accumulates in inflamed joints in an animal model of arthritis [91]. Several therapeutic strategies reported about NPs to improve T1DM are mainly based on insulin delivery systems, gene therapy and islet cell-targeting molecular therapeutics. Niu et al. [92], have shown that the human insulin gene can be transfected successfully by chitosan NPs in-vivo and in-vitro. Au-NPs-DNA functionalized conjugates used as an islet-targeting gene therapy have shown to be an effective and non-toxic transfection vehicle for islet cells by both in vitro and in vivo studies [93,94]. Jeong et al. [95], demonstrated surface camouflage of pancreatic islets using combination of cyclosporine and anti-CD4 monoclonal antibody (OX-38) along with PEGylation showed a highly improved synergistic effects on the inhibition of sensitized host immune reactions occurring against graft tissues. A study by Bhol et al. [96], silver nanocrystals were administered intracolonically at a dose as low as 4 mg/kg, and were effective to decrease the signs of colitis in a rat model of UC and was as effective as 100 mg/kg sulfasalazine.

Nanotechnology to correct deletion mutation (like thalassemia)

With the combined efforts of three Yale laboratories, researchers conducted the first demonstration of site-specific gene editing in a fetus, correcting a mutation that causes a severe form of anemia. The technique, which is developed in 2018, involves an intravenous injection of nanoparticles carrying a combination of donor DNA and synthetic molecules known as peptide nucleic acids (PNAs). The PNAs, which mimic DNA, bind to the target gene and form a triple helix — an aberration that triggers the cells' repair mechanisms. As part of this process, the healthy donor DNA, paired with the PNA in a nanoparticle, is used to fix the mutation. The researchers, made the nanoparticles with a degradable polymer and designed them small enough, 200 to 300 nanometers, to readily

accumulate in the liver of the fetus, where the stem cells are located before migrating to the bone marrow.

For the study, this gene-editing package was injected into the fetuses of mice as shown in figure (4). At four months after birth, the mice had been cured of thalassemia, an inherited defect in oxygen-carrying red blood cells. "The treated mice had normal blood counts, their spleens returned to normal size, and they lived a normal life span, whereas, the untreated ones died much earlier [97].

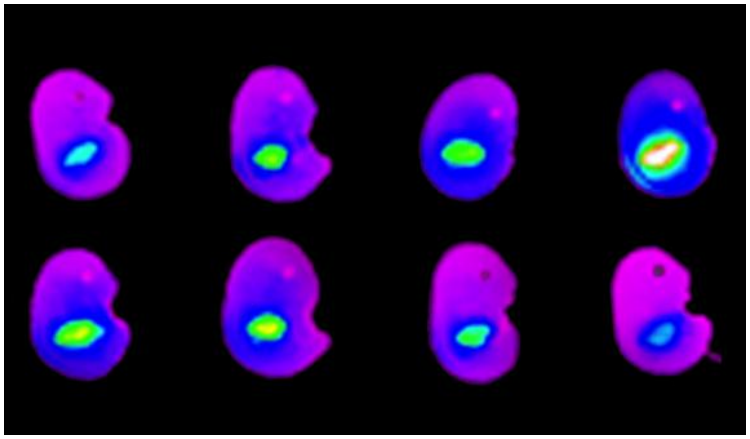

Figure 4. Distribution of nanoparticles in a litter of fetal mice after intravenous nanoparticle treatment. The intense green, yellow, and red areas show higher concentrations. The highest accumulation of nanoparticles in each mouse is in the fetal liver

Conclusion

Nanotechnology is a vast science, with a lot of advantages and some disadvantages that can be overcome by multiple, continuous and keen trials, until the best results gained.

In field of medicine, this technology opened new hopes to treat a lot of diseases that the traditional managements are not curative, so in other words, it reduced mortality for fatal diseases and improved life style for chronic morbid conditions.

The present review aimed is to illustrate nanoparticles types, and their clinical applications whether diagnostic or therapeutic, taking in consideration shape, size and consistency of the cover and core for each nanoparticle, as each type of them has its specific applications.

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