Detection of Epstein Barr Virus Infection in Reactive Arthritis Patients

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Abstract
Reactive arthritis (ReA) is an incendiary joint inflammation that occurs few days to weeks after a gastrointestinal or genitourinary infection. The etiology of the disease is not well-known. Therefore, the present study included 80 females and 25 males, divided into 51 patients with reactive arthritis and 54 healthy individuals as control group. The study involved the detection of serum levels of anti-rheumatoid factor and anti-cyclic citrullinated peptide antibodies (anti-CCP) as well as those of CRP and C3 in all subjects. In addition, EBV levels were detected by Real Time-PCR technique. The results showed significantly increased levels ($P < 0.05$) of CRP, C3 and anti-CCP Ab in ReA patients’ group compared to the healthy control group ($505.42 \pm 402.94 \text{ versus } 255.62 \pm 135.5 \text{ U/ml}$, $61.20 \pm 100.64 \text{ versus } 20.43 \pm 47.63 \text{ ng/ml}$ and $35.11 \pm 30.0 \text{ versus } 6.82 \pm 14.01 \text{ pg/ml}$, respectively). Also, the RF results demonstrated a significantly increased percentage in ReA patients’ group compared to a healthy control group ($61.11 \text{ versus } 37.25 \%$). While, the molecular study showed a non-significant increase in the percentage of EBV in ReA patients’ group compared to a healthy control group ($17.65 \text{ versus } 12.69 \%$). The results of this study lead to suggest that the immunological markers used may play a role in the development of ReA disease, while there was a non-significant association between EBV infection and ReA disease development.

Keywords: Reactive arthritis, ReA, Epstein–Barr virus, anti-CCP antibodies, CRP, C3 and RT-PCR.

التحري عن الإصابة بفايروس ابشتاين بار في مرضى إلتهاب المفاصل التفاعلي

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الخلاصة:
التهاب المفاصل التفاعلي هو انتهاض رفيع بالعظام بعد بضعة أسابيع من الالتهاب المعي أو انتهاض مثار بالمنطقة المحيطة. يعتمد نتائج الإحصائيات على فحص المريض، توجد دراسة حالية 80 أشخاص و25 نرجم، تحتوي على 54 شخصًا ويلعبون كجهاز ميتري، وقد تم الكشف عن أضداد العامل الروماتيزي، وأضداد البيريد المشتهرة للورماء (CCP)، كمساهمة رئيسية لكل من CRP والضمن الثالث لجميع فيتالي الدراسة. بالإضافة إلى الكشف عن وجوه فريش ببرونس ابشتاين بار (EBV) في عينات الدراسة باستخدام تقنية تفاعل البصمة المنشورة الخطي، أظهرت نتائج الدراسة الحالية زيادة معنوية في منسوب كل من بروتيني التفاعل (CRP) والضمن الثالث وأضداد

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In the second to fourth decade of life, EBV usually spreads to infectious mononucleosis (IM) [13]. EBV infects almost every person in adulthood [14]. Moreover, this virus has been related to a broad spectrum of malignancies, including post-transplant lymphoproliferative diseases (PTLDs), nasopharyngeal carcinoma (NPC), Hodgkin lymphoma, and gastric carcinoma (MS) [12, 14]. EBV usually spreads through salivation, then reaches the tonsils’ epithelium and starts the lytic period of illness that involves the reproduction of infections [15]. The present study aimed to find the relationship between EBV infection and the development of reactive arthritis.

Samples and methods
This case – control study was conducted in Baquba Teaching Hospital at Baquba City, Diyala Province, Iraq. In this study, 25 males and 80 females were involved, including 51 samples (8 males and 43 females) with ReA that was diagnosed by the physicians, as well as 54 samples (17 males and 37 females) without ReA symptoms as a healthy control group. The age of participants was between 16 - 80 years. The period of samples collection was three months from January 15th to April 4th 2019.

Blood sample collection
Five milliliters of venous blood was collected from each individual in this study. Each sample was divided in two sterile test tubes (gel tube and EDTA tube). The blood in the first tube was left for clotting, then centrifuged for 10 minutes at 2500 rpm. The serum was aspirated by pipette and stored in a clean tube at -20°C until used in the immunological tests. While the blood in the anticoagulant tube was stored at -20°C until used for the detection of EBV.

The evaluation of immunological and molecular markers
Rheumatoid Factor (RF) (Sino Gene Clone Biotech, China), Complement component 3 (C3) (Sino Gene Clone Biotech, China), anti- Anti-Cyclic Citrullinated Peptide antibody (anti-CCP Ab) (Sino Gene Clone Biotech, China) and C-Reactive Protein (CRP) (Sino Gene Clone Biotech, China) were
estimated by ELISA technique. In addition, the molecular detection of EBV was performed by Real Time-Polymerase Chain Reaction (RT-PCR) technique (QIAGEN GmbH, USA and Applied Biosystem ThermoFischer Scientific Ltd., USA).

**Statistical analysis**

The statistical analyses were performed on mean, standard deviation, and percentage data by employing student’s T-test and Chi-square test with an IBM SPSS computer software (IBM Statistical Package for Social Sciences version 17) in association with Microsoft Excel 2010. Probability values of less than 0.05 were considered significant. The analysis of Real Time-PCR data was conducted using Rotor-Gene Q Series Software 2.3.1 (Build 49) 2013, QIAGEN GmbH.

**Results**

The results revealed a significant increase ($P<0.05$) in the frequency of RF in ReA group compared to the controls. There were 33 (61.11%) positive samples out of a total of 51 ReA patients’ samples. While in the control group, 19 (37.25%) positive samples were found out of a total of 54 samples (Table-1).

**Table 1-Serum RF frequency in ReA patient group compared to control**

| Groups     | No. | RF frequency (%) | $P$-value |
|------------|-----|------------------|-----------|
|            |     |                  |           |
| Control    | 51  | 19 (37.25)       | 0.015     |
| Patients   | 54  | 33 (61.11)       |           |

The data are presented as No. (%). The differences are significant at $P<0.05$. Moreover, there was a significantly increasing level ($P<0.05$) of CRP in ReA patients’ group compared to the controls (505.42 ± 402.94 vs. 255.62 ± 135.85 pg/ml, respectively) (Table-2).

**Table 2-CRP serum level in ReA patients group compared to control group**

| Groups     | No. | CRP level pg/ml | $P$-value |
|------------|-----|-----------------|-----------|
|            |     | Mean            | Std. Deviation | |
| Control    | 54  | 255.62          | 135.85    | 0.007 |
| Patients   | 51  | 505.42          | 402.94    |     |

The data are presented as mean ± SD. The differences are significant at $P<0.05$. Also, the results of complement 3 (C$_3$) shown in Table- 3 revealed a significantly increased level ($P<0.05$) in ReA patients group compared to control group (61.20 ± 100.64 vs. 20.43 ± 47.63 ng/ml, respectively).

**Table 3-Levels of serum C3 complement in studied groups**

| Groups     | No. | C$_3$ level ng/ml | $P$-value |
|------------|-----|-------------------|-----------|
|            |     | Mean              | Std. Deviation | |
| Control    | 54  | 20.43             | 47.63     | 0.009 |
| Patients   | 51  | 61.20             | 100.64    |     |

The data are presented as (mean ± SD). The differences are significant at $P<0.05$. In addition, the results in Table- 4 show a significantly increased level ($P<0.05$) of serum anti-CCP levels in ReA patient’s group compared to control group (35.11 ± 30.00 vs. 6.82 ± 14.01 U/ml, respectively).
Table 4-Serum anti-CCP antibody level in ReA patients’ group and control group

| Groups | No. | Anti-CCP Ab level U/ml | P-value |
|--------|-----|------------------------|---------|
|        |     | Mean | Deviation |         |
| Control| 54  | 6.82 | 14.01     | 0.003   |
| Patients| 51  | 35.11| 30.00     |         |

The data are presented as (mean ± SD). The differences are significant at P<0.05.

The result of EBV detection by Real time-PCR technique revealed that nine samples were positive to EBV in ReA patients’ group, whereas seven samples were positive in the control group. It showed the positively for EBV, presented in the CT between 20.5 to 21 compared as positive control as shown in Figure- 1 and Table -5. In addition, the odd ratio was 1.44, while fishers’ exact probability referred to a non-significant difference between ReA patient and control groups, as reflected in the similar passivity results in both groups, possibly due to the small sample size of the selected groups. The present results disagree with those of a previous study which reported that EBV was highly spread among the population worldwide, 90% of which were carrying the virus [12].

Table 5-EBV infection percentage between the studied groups

| EBV infection status | Groups | Control | Patients | Total |
|----------------------|--------|---------|----------|-------|
| Positive (%)         |        | 7 (12.96) | 9 (17.65) | 16 (15.24) |
| Negative (%)         |        | 47 (87.04) | 42 (82.35) | 89 (84.76) |
| Total (%)            | 54 (100.0) | 51 (100.0) | 105 (100.0) |
| Odd ratio            | 1.44   |          |          |       |
| Fisher’s exact probability value | 0.592 |          |          |       |

Figure 1-Real Time-PCR results for positive samples to EBV. The curve shows the positivity for EBV, presented as CT values between 20.5 and 21, as compared to the positive control.

Discussion

The incidence studies of EBV and its relationship to ReA disease are very few in the world and in Iraq, despite the high incidence rate of the virus among the population worldwide [12]. Therefore, the present study was designed to investigate the relationship between EBV infection and ReA in the Iraqi population. The present results revealed that 17.65% of the patients had positive results for EBV. In addition, the virus was found in the control group (12.96%). The sample that were found positive to
EBV might represented the reactivation stage of the virus, whereas the other samples might have the virus but in the latent phase, making it undetectable.

The results of RF frequency percentage showed a significant increase ($P<0.05$) in ReA patients group compared to control group (61.11% vs. 37.25%, respectively). These results agree with those published by Francesca et al. (2013), who reported that RF is one of the diagnostic factors for ReA [16]. While, Newkirk (2002) reported that RF seropositive percentage was 5% in patients with spondylitis diseases [17].

CRP is an acute phase protein of hepatic origin; it is a biomarker commonly used for the assessment of inflammatory reactions [18]. Thus, CRP is one of the acute phase proteins that increase in level during systemic inflammation, bacterial infection, autoimmune disease, and cancer [19, 20]. In the present study, the results of CRP showed a significantly increasing levels in ReA group compared to control group, these results agree with those of Kim et al. (2009) and Li et al. (2010) who reported that CRP level increased in ReA patients [21, 22]. Moreover, the results of $C_3$ level showed a significantly increased level ($P<0.05$) in ReA patients’ group compared to control group (61.20 ± 100.64 vs. 20.43 ± 47.63 ng/ml). These results agree with those of Salloom et al. (2018) who found that levels of some immunological markers, as in those of innate immunity, were increased [23]. Also, the present results showed that the standard deviation was higher than the mean level of $C_3$, which is due to the highly increased level of $C_3$ in some ReA patients. The complement system is an important contributor to various human diseases, such as autoimmune, inflammatory and infectious diseases [24].

The same result appeared when anti-CCP Ab level was assessed; there was a significantly increased level of anti-CCP Ab ($P<0.05$) in ReA patients’ group compared to control (35.1.1 ± 30 vs. 6.82 ± 14.01 U/ml). These results confirmed those of Singh et al. (2018) who referred to the association of the increased level of anti-CCP Ab and ReA incidence [25]. Hence, the anti-CCP antibodies were associated with the severity of autoimmune disease. However, the present results disagree with those of De Rycke et al. (2004) and Korkmaz et al. (2006) who demonstrated the positive correlation between anti-CCP antibodies and extra-articular manifestations [26, 27]. Anti-CCP antibodies were recognized as particular RA markers and their test is included in the updated ReA diagnostic classification criteria [28].

Finally, the results of EBV detection by RT-PCR reported that 9 (17.65%) ReA cases were positive for EBV infection, while 7 cases were positive in control group. This study agree with that of Jassim et al. (2015) which reported that EBV may play a role as a triggering factor in the pathogenesis of reactive arthritis [29]. Patients with ReA have increased anti-EBV antibody levels in their sera compared to healthy subjects [30]. While, López et al. (2016) pointed out a positive relationship between inflammatory arthritis and EBV [31]. In contrast, the results of Westergaard et al. (2015) disagreed with the present results, since they confirmed that infection with the EBV was not a triggering factor for arthritis [32]. Several studies showed elevated humoral and cellular anti-EBV immune responses in rheumatoid arthritis patients, indicating that the virus may be associated with the autoimmune dysfunction in patients with RA [33]. Several other studies also suggested that EBV may cause the development of anti-CCPs, which are extremely specific to RA [34].

Conclusions

This study concludes that the immunological markers may play a role in the development of ReA disease, while there was a non-significant association between EBV infection and ReA disease development.

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