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The Relationship between Gene Expression of Human Leukocyte Antigen – DR4 and Enteric Bacteria as Environmental Factor in Iraqi Patients with Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is a complex autoimmune disorder that is shaped by the interplay of genetic predisposition and environmental triggers, which collectively contribute to the onset and progression of the disease. This study pursued three key goals: first, to estimate the expression levels of the Human Leukocyte Antigen – DR4 (*HLA DR4*)gene; second, to identify the predominant enteric bacteria in rheumatoid arthritis (RA) patients and healthy controls; and third, to examine the association between HLA-DR4 gene expression and the identified bacterial profiles. The case-control study involved 100 blood specimens and 100 stool specimens were collected from 50 RA patients and 50 healthy individuals. RNA was extracted and cDNA was synthesized, then by RT -PCR was used to detect *HLA DR4*gene expression. Also, Stool specimens were collected and analyzed using culturing, biochemical tests, antibiotic sensitivity tests, and 16S rRNA PCR. This process helped evaluate the distribution of enteric bacteria and detect the bacterial Cefotaximase-M (CTXM) gene. The results indicated that *HLA DR4* gene expression was highly expressed in RA patients compared with the control. *Escherichia coli* is the predominant enteric bacteria in the stools of patients, and they carry the highest level of the CTXM antibiotic resistance gene, other enteric bacteria were diagnosed also; which included *Staph aureus, Shigella sonnei, Klebsiella pneumonia, Proteus marbilus,* and *Pseudomonas aeruginosa* while Controls enteric bacteria include *E.coli, Lactobacillus spp., Enterococcus spp*., and *Bifidobacterium spp*., among the total sample, there was no significant difference in mean between *HLA DR4* gene expression and the bacterial distribution. The present study indicates that the *HLA DR4* gene and enteric microbial dysbiosis are involved in RA pathogenesis. These elements are expected to be crucial in identifying potential biomarkers and predictors that can aid in the diagnosis of the condition.

Keywords: Enteric bacteria, *HLA DR4* gene expression, *HLA DR4* gene-Enteric bacteria interactions, Rheumatoid arthritis.

العلاقة بين العامل الوراثي HLADR4 والبكتيريا المعوية كعامل بيئي في مرضى التهاب المفاصل الروماتويدي

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الخلاصة

التهاب المفاصل الرثوي (RA)، وهو احد أمراض المناعة الذاتية التي تتأثر بعوامل الخطوره الوراثية والبيئية ،التي تلعب دورًا مهمًا في تطور المرض. هناك ثلاثة اهداف للدراسة الحالية وهي: أولا: تقدير مستويات التعبير عن جين مستضد الكريات البيضاء البشرية(HLADR4) ؛ ثانيا: تحديد البكتيريا المعوية السائدة لدى مرضى التهاب المفاصل الرثوي ؛ وثالثًا، فحص العلاقة بين التعبير عن جين(*HLA DR4*) بالبكتيريا المعزوله. حيث شملت الدراسة 100 عينة دم و100 عينة براز تم جمعهم من 50 مريضًا بالتهاب المفاصل الرثوي، و50 فردًا سليمًا. وتم استخراج الحمض النووي الريبي (RNA)، ونسخ(cDNA) وثم استخدام تقنية RT–PCR للكشف عن التعبير الجيني لـ *HLA DR4*. كما تم جمع عينات البراز تليها الزراعة وإجراء اختبارات الكيمياء الحيوية واختبار الحساسية للمضادات الحيوية و 16 sRNA PCR لتقييم مدى انتشار البكتيريا المعوية ووجود جين *CTXM* البكتيري بواسطة PCR. ان نتائج التعبير الجيني *HLA DR4* كانت أكثر إيجابية في مرضى التهاب المفاصل الرثوي مقارنة بالاصحاء. الإشريكية القولونية هي البكتيريا المعوية السائدة في براز المرضى، وهي تحمل أعلى مستوى من جين مقاومة المضادات الحيوية *CTXM*، كما تم تشخيص البكتيريا المعوية الاخرى في المرضى ؛ والتي شملت العنقوديات الذهبية، والشيجيلا سوني، والكلبسيلة الرئوية، والبروتيوس ماربيلوس، والزائفة الزنجارية، في حين شملت البكتيريا المعوية الضابطة الإشريكية القولونية، والعصيات اللبنية، والمكورات المعوية، والبيفيدوباكتيريوم . بين العينة الإجمالية، لم يكن هناك فرق كبير في المتوسط بين التعبير الجيني *HLA DR4* والتوزيع البكتيري. تشير نتائجنا إلى أن جين *HLA DR4* والتغيير في المحتوى الميكروبات المعوية لهما دور في التسبب في التهاب المفاصل الرثوي. ويمكن اعتبار هذه العناصر كمنبئات محتملة في تشخيص المرض.

1. Introduction:

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by persistent inflammation in the synovial tissue surrounding the joints. This inflammatory process leads to the erosion of bones and cartilage, resulting in joint destruction [1]. The development of this condition is influenced by a combination of both environmental and genetic factors, which together contribute to an individual's vulnerability to the disease [2]. RA is widely dispersed, with a prevalence of around 0.5% to 2%. Additionally, it is more commonly found in women, smokers, and those with a familial history of the disease [3]. Additionally, RA can develop when a genetically predisposed individual encounters an environmental factor, primarily microbial . Numerous studies have highlighted the significant contribution of the gut microbiota in the development of rheumatoid arthritis (RA) [4]. This phenomenon is primarily linked to several mechanisms, including the generation of pro-inflammatory metabolites, disruption of the intestinal mucosal barrier, and molecular mimicry with self-antigens [5]. Also, a genetic marker, *HLA DR4*, a human leukocyte antigen in white blood cells, indicates a potential risk for RA. This marker plays a crucial in distinguishing between the body's cells and foreign substances. However, when *HLA-DR4* fails to differentiate between the two, it may mistakenly attack the body's cells [6]. Furthermore, the onset of RA is not restricted to individuals who are *HLA-DR4* positive. Instead, it can be influenced by host-related factors and environmental elements like smoking, diet, periodontal disease, gastrointestinal problems, psychological stress, gut dysbiosis, and age-related immune system alterations [7]. There are three potential ways the microbiota may contribute to the onset of rheumatoid arthritis. First, localized inflammation within the intestines may contribute to systemic inflammation in individuals who possess a genetic susceptibility to rheumatoid arthritis (RA). Second, molecular mimicry can occur when specific bacterial proteins possess amino acid sequences that resemble self-antigens. Lastly, compromised intestinal barrier function can play a role in the development of rheumatoid arthritis [8].

2. Materials and Methods

The present study involved 100 specimens of stool and 100 specimens of blood from (50) RA patients of different ages (21-70 years), all under treatment, and (50) control (age range 18-70) who participated voluntarily in this study. All patients were examined and diagnosed by a rheumatologist. In addition to blood and stool sampling, a questionnaire was also administered, composed of several questions, including information about the participants. All samples were collected from Al-Zahraa Teaching Hospital, Al-Karamaa Teaching Hospital, the Rehabilitation Center of the disabled, and prosthetics also from Rheumatologists private clinics in the governorate of Wasit, Iraq between July 2023 and June 2024. A total of 2.5 mL of venous blood from a vein of both the control group and the patient group. The blood was placed in EDTA tubes (Human RNA extraction was done by using the GENEzol™ TriRNA Pure Kit, and Conversion of mRNA to Complementary DNA (cDNA) was done by using One-Step gDNA Removal and cDNA Synthesis Super Mix with PCR then RT-PCR) using *HLA DR4* primer with sequences. F: GTTTCTTGGAGCAGGTTAAAC R: CCGCTGCACTGTGAAGCTCT [8], and housekeeping gene (*GAPDH*) primer sequences F: GAAATCCCATCACCATCTTCCAGG, R: GAGCCCCAGCCTTCTCCATG [9]. Real-time PCR was carried out to detect relative *HLA DR4* gene expression. Stool specimens were collected and transported to the lab by Carry Blair (transport swab)from Yancheng Huida in China for culturing in different Culture Media to identify enteric bacterial types in patients and control. Then, the biochemical test and antibiotic sensitivity test were done as 16sRNA test by PCR using a primer with sequence F: GGGAGCGAAAATCCTG, R: AGTACAGGTAGACTTCTG [10]. Furthermore, *E coli* virulence *CTXM* gene detection was done by using a primer with sequence F: TTA GGA ART GTG CCG CTG YAb, R: CGA TAT CGT TGG TGG TRC CAT [11], by PCR. Then Agarose Gel Electrophoresis Technique was applied to the DNA sample for visualization under UV light.

Ethical approval

This research was approved by the Council of College of Medicine, University of Wasit in July 2023 and by the "Wasit Health Directorate (1201)" on 24/7/2023. Prior to sampling process, all individuals involved in the study were informed about its objectives, provided their agreement and verbal consent was secured from each participant. The study was conducted following the principles of the Declaration of Helsinki.

Exclusion Criteria

1- Patients who have declined to take part in the study and patients who have not adhered to their prescribed medications or have discontinued treatment within the past three months.
2- Study including adult patients (>16), children and young (<16) are excluded because they are affected by juvenile rheumatoid arthritis.
3- Pregnant women

Statistical analysis

The study results were displayed as mean ± standard error (M±S.E.), and statistical analysis was carried out using the SPSS software (version 26) to compare parameters between two groups. Median, and Interquartile range (IQR) if they are continuous numerical. Both testes Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess if the continuous variables were normally distributed or not. The chi-square test or Fisher's Exact Test was used to assess the presence of an association between categorical variables. To assess the differences between two numerical variables, the independent samples t-test or Mann-Whitney U Test were used. One-way Analysis of Variance (ANOVA) or Kruskal-Wallis Test was used according to a type of data distribution, with statistical significance set at p-value <0.05.

3. Results

3.1 Bacterial types and distribution

All the study specimens were found to predominantly contain *E. coli* (100%) and there are 80% of them with only *E. coli*, and the remaining 20% of patients were found to have *E.coli* and another type of bacteria(*Staph aureus, Shigella sonnei, Klebsiella pneumonia, Proteus marbilus* and *Pseudomonas aeruginosa*). In contrast, all specimens from the control group were found to have *E.coli* and some *lactobacillus spp., bifidobacteria spp., and enterococcus spp*. bacteria, which indicates a significant difference from the patient group. In addition to *E. coli* which was present in all patients, other bacterial species were also detected in a subset of the patient group. *Staph aureus* was found in 8 patients followed by *Proteus marbilus* in 5 patients, and only *Shigella sonnei, and Klebsiella pneumonia* was detected in 3 patients that demonstrated their association with RA condition as listed in Table 1. The 16sRNA for *E coli* shown in Figure (1).

Table 1: Bacterial types and bacterial distribution detection among the study specimens.

Bacteria	Frequency	Percentage	Patient(n=50)	Control(n=50)	P-value
Only *Escherichia. coli*	80	80%	30 (60%)	50 (100%)	<0.001
Staph aureus	8	8%	8 (16%)	0 (0%)	
Shigella sonnei	3	3%	3 (6%)	0 (0%)	
Klebsiella pneumonia	3	3%	3 (6%)	0 (0%)	
Proteus marbilus	5	5%	5 (10%)	0 (0%)	
Pseudomonas aeruginosa	1	1%	1 (2%)	0 (0%)	

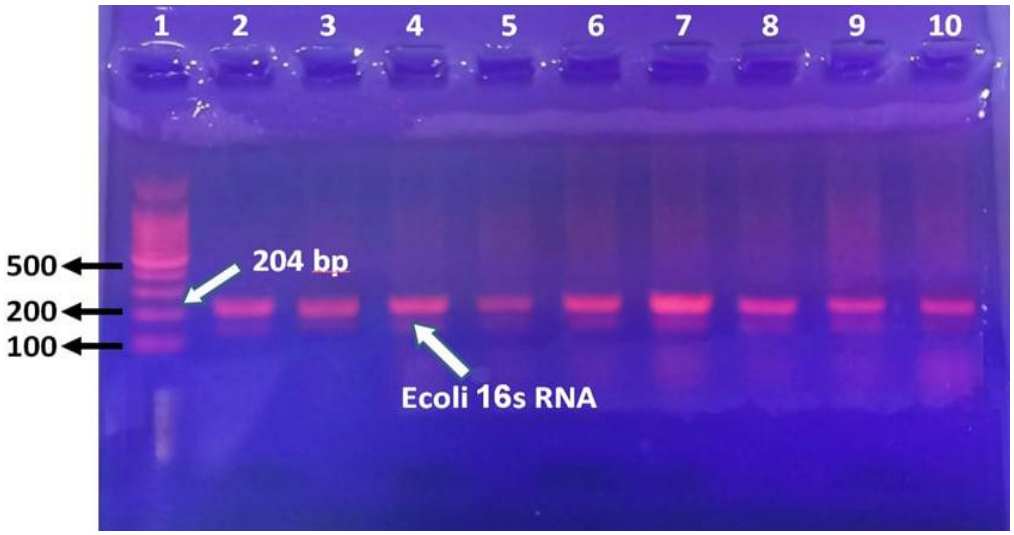

Figure 1: *E coli* 16sRNA Gene, 204bp was shown in agarose gel for stool specimens from RA patients.

3.2 Sensitivity test for different antibiotics.

A significant association was observed in *E coli* culture and sensitivity responses related to different antibiotics with a P-value less than 0.001. The specimens from our study revealed that patient's resistance to Ampicillin antibiotic 86 %, Trimethoprim 78%, Rifampin 60%, sulfamethoxazole 76%, Ceftazidime 74%, lincomycin 70%, Clindamy 58%, colistin 44%, cefixime 42%, Meropenem 34%, Gentamicin 30%, Aztreonam 14%, Ciprofloxacin 8%, vancomycin 6%, Amikacin 4% and Imipenem 2%. While that control specimens in the study showed resistance to Ampicillin antibiotic 22 %, Trimethoprim 14%, Rifampin 12%,

sulfamethoxazole14%, Ceftazidime 6%, lincomycin 12%, Clindamy 14%, colistin 16%, cefixime 6%, Meropenem 4%, Gentamicin 2%, Aztreonam 4%, Ciprofloxacin 4%, vancomycin 2%, Amikacin 2% and Imipenem 0%. The sensitivity result for different antibiotics is seen in Table(2)

Table 2: Antibiotic susceptibility test for *E coli* specimens

Antibiotic	Clinical D E.C (patients=50)						P value	Clinical DEC (control= 50)						P value
	R	%	I	%	S	%		R	%	I	%	S	%	
Amikacin	2	4	2	4	46	92		1	2	0	0	49	98	
Ciprofloxacin	4	8	3	6	43	86	<0.001	2	4	1	2	47	94	<0.001
lincomycin	35	70	0	0	15	30		6	12	11	22	33	66	
Ampicillin	43	86	6	12	1	2		11	22	7	14	32	64	
Meropenem	17	34	0	0	33	66		2	4	0	0	48	96	
Aztreonam	7	14	1	1	42	84		2	4	0	0	48	96	
Rifampin	38	76	7	14	5	10		6	12	2	4	42	84	
Clindamycin	29	58	13	26	8	16		7	14	6	12	37	74	
Ceftazidime	37	74	0	0	13	26		3	6	0	0	47	94	
cefixime	21	42	0	0	29	58		3	6	1	2	46	92	
Gentamicin	15	30	4	8	31	62		1	2	6	12	43	86	
Imipenem	1	2	4	8	45	90		0	0	0	0	50	100	
Trimethoprim	39	78	2	4	9	18		7	14	7	14	36	72	
sulfamethoxazole	38	76	0	0	12	24		7	14	3	6	40	80	
vancomycin	3	6	2	4	45	90		1	2	0	0	49	98	
colistin	22	44	0	0	28	56		8	16	0	0	42	84	

*R= resistance, *I= intermediate response, *S=sensitive, *D E.C= Disk approximation test of *E coli* antibiotic sensitivity

3.3 Association of study groups with CTXM I *E. coli*

There was a significant association between the study groups (patients and controls) regarding the presence of *CTXM I* of *E. coli* (P-value <0.001). Even though all patients and controls had positive *E.coli*, most of the patients (86%) had *E coli* with *CTXM I*, and most of the controls (78%) had negative *E.coli* with CTXM I. These findings are listed in Table (3) and *E coli* CTXM Gene 688bp in Agarose Gel for the RA patient's stool sample, as shown in Figure (2).

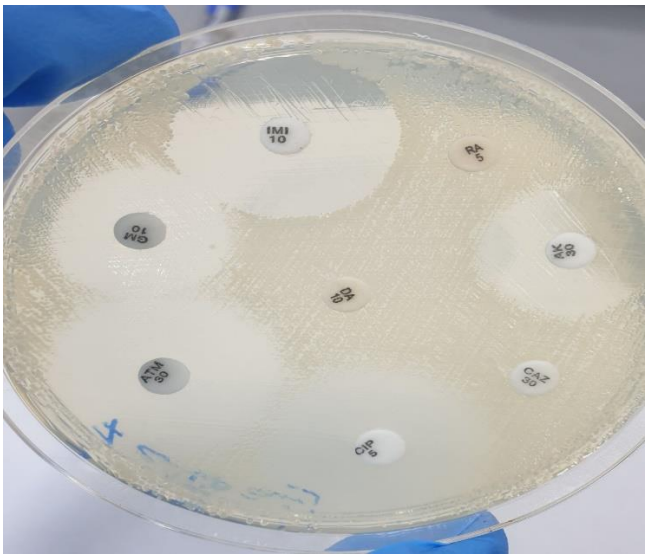

Figure 2: Antibiotic sensitivity test of *E coli* by disk approximation test.

Table 3: Association of study groups with CTXM I *E. coli.*

CTXM I *E. coli*	Patient	Control	Total	P-value
Positive	43(86%)	11(22%)	54(54%)	<0.001
Negative	7(14%)	39(78%)	46(46%)	

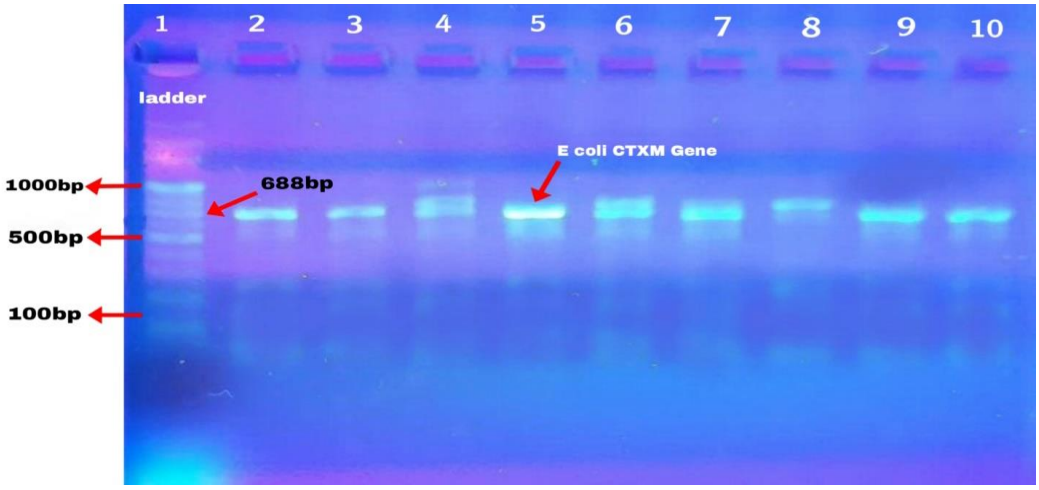

Figure 3: *CTXM* Gene of *E. coli.* (688bp) was shown in Agarose Gel for stool Specimens from RA patients. DNA Ladder from Promega 100-1500 bp.

3.4 Gene expression of HLA-DR4 in patients and control

In the study, approximately 62% of patients with RA exhibited positive gene expression, compared to only 20% of the non-diseased control group. Furthermore, there is a statistically significant association between the presence of gene expression and the disease status with a P-value less than 0.001. Furthermore, overexpression of HLA-DR4 in patients with RA (1.161-fold) compared to standard controls, exhibited potential implicates in the pathogenesis of the disease. The gene expression, or relative quantification (R.Q.) value in this study, is calculated using the formula RQ = 2ˆ(–ΔΔCT). Initially, the average cycle threshold (C.T.) or cycle quantification (C.Q.) values are obtained from a real-time PCR apparatus for each specimen. Subsequently, The ΔCT value is calculated for both patient and control specimens using the formula: Δ CT = C.T. (Target gene) – C.T. (reference gene). The ΔΔCT value is then calculated by subtracting the Δ CT of the control specimens from the Δ CT of the patient specimens, expressed as ΔΔ CT = Δ CT (patient sample) – Δ CT (control sample). Finally, the gene expression or R.Q. is derived from the fold change using the equation Fold change = $2^{-(\Delta\Delta CT)}$.

Table 3: The result of analyzing Real-Time PCR data for gene fold

Variable	HLADR4 Gene	GAPDH	Δct	$2^{-}\Delta ct$	experimental	fold
Av. Pat.	**22.273**	**14.984**	**7.288**	**0.00639872**	**0.00639/0.00551**	**1.161**
Av. Con.	**23.3408**	**15.8392**	**7.5016**	**0.00551815**	**0.00551/0.00551**	**1**

Also, in this study, patients with RA had a statistically significant difference in HLADR4 gene expression from the controls (P-value =0.045) by having a slightly higher HLADR4 mean (1.21±0.35) than controls (1.0±0.14). The median and IQR were also different; it was 1.28 (0.59) in the patient and 0.96 (0.04) in controls and are listed in Table (4) and as shown in Figures 3 and 4.

Table 4: The association and difference in HLADR4 gene expression between patients and control groups (n=100).

Variable	PatientNo.%	ControlNo.%	Total	P-value
Positive gene expression	**31(62%)**	**10(20%)**	**No. 41(41%)**	**<0.001**
Negative gene expression	**19(38%)**	**40(80%)**	**No. 59(59%)**	
Mean of folds ± StandarDeviation	**1.21±0.35**	**1.01±0.14**		**0.045**
Min-max	**0.68-2.30**	**0.80-1.32**		
IQR	**0.59**	**0.04**		

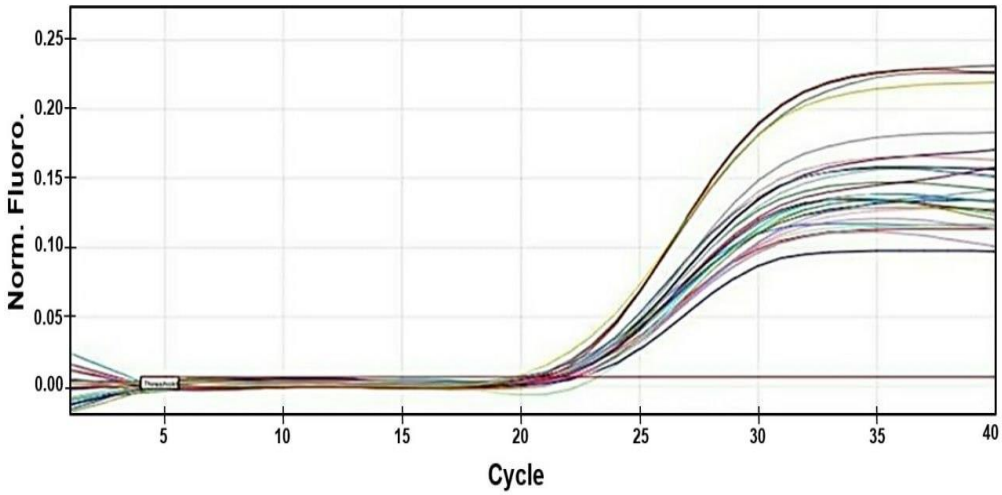

Figure 4: determination of *HLA DR4* gene expression by RT-Q real-time PCR, the curves represent the patient's gene expression, with Average patient Dr4 gene ct 22.273and total fold 1.161.

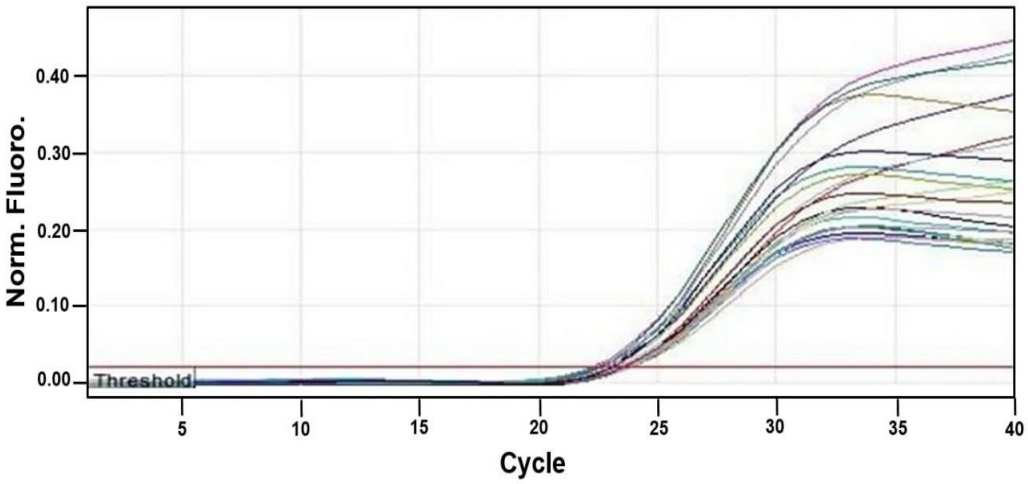

Figure 5: determines *HLA DR4* gene expression by RT-Q real-time PCR melting curves analysis; the curves represent the control gene expression, with average control *HLA DR4* gene ct 23.3408 and total fold 1.

3.5 Association of *HLA- DR4* gene expression with bacteria

The total specimens (n=100) showed no significant difference in fold mean *HLA DR4* gene expression between the bacterial distribution with P-value=0.178, as summarized in Table (5).

Table 5: Mean *HLA DR4* gene expression difference with bacteria among the study sample

Bacteria	No.	Mean	Standard Deviation	P-value
Only *Escherichia. coli*	**80**	**1.12**	**0.28**	**0.178**
Staph aureus	**8**	**1.08**	**0.34**	
Shigella sonnei	**3**	**1.23**	**0.35**	
Klebsiella pneumonia	**3**	**1.14**	**0.25**	
Proteus marbilus	**5**	**0.94**	**0.27**	
Pseudomonas aeruginosa	**1**	**0.73**	**-**	

4. Discussion

The current study reveals changes in the gut microbiota of RA patients. A comparison of the gut microbial populations between healthy and affected individuals pointed out the role of *Escherichia coli* and some pathogenic types of bacteria (*Staph aureus*, *Shigella sonnei, Klebsiella pneumonia, Proteus marbilus,* and *Pseudomonas aeruginosa*)in the pathogenesis of RA. These alterations in gut microbiota may arise due to host-specific elements like health conditions (infections, inflammation), genetic background, and lifestyle choices. However, environmental factors like xenobiotics (antibiotics, medications, food additives), dietary habits (high sugar, low fiber), and hygiene practices [12]. The result agrees with Sun,2019. Their study demonstrated notable distinctions in the diversity and composition of the microbiome between patients with rheumatoid arthritis (RA) and healthy individuals [13]. Additionally, the misused antibiotic-induced disturbances of the human microbiota have been associated with the onset of RA and the development of chronic autoimmune conditions [14,15]. Also, the current elevated level of the *E coli CTXM I* gene demonstrates the dominance of *E coli* antibiotic resistance as the primary bacteria in the gastrointestinal tract of patients undergoing treatment. The CTXM I *E. coli*, which is one of the virulence bacterial genes, a most prevalent form of extended-spectrum beta-lactamase (ESBLs) gene that was present on a plasmid and was transmitted among members of the *Enterobacteriaceae* family, with a particular prevalence in *E. coli* [16]. The current result agrees with Tawfick, 2021. They have found that when human gut commensal *E. coli* acquire antimicrobial resistance (AMR) genes, that indicates a possible health risk for humans, and *E. coli* bacteria residing in the gut could serve as a source of AMR genes that have the potential to spread to different bacterial strains [17]. Furthermore, arthritis is often preceded by gut dysbiosis, although the precise mechanisms by which microbes initiate immune responses in distant areas are still not completely understood. The present study found that the *HLA DR4* gene expression is strongly associated with RA. The finding was similar to that reported in the Kurdish region in the North of Iraq [18], and Iran [19]. Conversely, a study on Mexican American found populations showed no significant correlation between *HLADR4* and RA susceptibility [20]. The primary function of this marker is to distinguish between an individual's cells and external pathogens. However, in cases where HLA-DR4 fails to differentiate between self and non-self accurately, it may attack the body's cells [6]. However, the mechanism of action of *HLA-DR4* in rheumatoid arthritis susceptibility is unclear, although genetic factors influence RA onset and its severity [8]. In addition, the genetic factor could potentially impact the expression of anti-cyclic citrullinated peptide (anti-CCP) and rheumatoid factor (RF) [21]. Individuals carrying this gene were at a higher risk of developing RA compared to those who do not possess it [22]. In the current study, no statistical association was found between *HLA DR4* gene expression and bacteria type in the gut of RA patients. The current result disagrees with [23]. This finding contrasts with previous research, which indicated a connection between gut microbiota and host genetic risk for RA. The interaction between host genetic factors such as MHC and intestinal microbiota and its impact on the development of rheumatoid arthritis is difficult to study in humans because of the high

variability of genetic factors and diet and geographical location that can influence the gut microbiome composition [24]. However, the genetic factor and the environmental factor remain two risk factors that play an essential role as a cause of the disease, so when a patient has human leukocyte antigen (HLA) HLA-DR4 and is affected by an environmental agent, then there is a chance of RA [25-27].

Conclusions

The *HLA DR4* gene expression is overexpressed in RA patients and may be involved in the pathogenesis of the condition and demonstrates microbiota dysbiosis in RA patients and considers these elements as potential predictors in diagnosing the condition. Furthermore, antibiotic misuse/overuse can induce disturbances of the human microbiota which has been associated with the onset of RA and the development of chronic autoimmune conditions. It poses a significant risk to the elderly. Regulating antibiotic usage and restoring the beneficial organisms in the patient's gut is essential.

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Conflict of interests

The authors declare no conflicts of interest among them.

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