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Molecular Assessment of Gene Expression for Interleukine-4 and Interleukin-17 among Patients with Human Bronchial Asthma by Conventional and Real Time q-PCR Techniques

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Abstract: ***This study aimed to detect IL 4 and IL 17 by conventional and RT PCR. DNA-based techniques have been further simplified benefiting from the introduction of PCR the result of this estimation revealed that the amplified DNA (PCR product) was 710 bp for IL-4 (50 voltage for 1 hour) and the presence of IL-4 was 85% from total samples, (PCR product) was 344 bp for IL-17 on agarose gel (1%) The presence of IL-17 was 70 % from total samples. RT PCR IL-4 show peak between 84.5 – 85.2 and IL-17 peak between 86.5-87.5.***

Keywords: conventional and Real Time q-PCR techniques, human bronchial asthma, interleukins

1. Introduction

Asthma is a complex respiratory disease in which genetic predisposition, environmental and immunological influences interfere with each other (**Edwards et al., 2012).** It is considered one of the most prevalent chronic diseases, affecting approximately 300 million individuals (Masoli et al., 2004) and causing an estimated 250, 000 deaths each year (Bateman et al., 2008). In addition, it is projected that by 2025, the global asthma burden will rise by 100 million people due to a growing Westernized lifestyle and urbanization in developing countries (Masoli, et al., 2004). The 'hygiene theory' was originally attributed to an increase in the prevalence of allergic diseases, including asthma, indicating that decreased exposure to microbes during the first years of life plays a role in the development of allergic diseases (Strachan, 1989, 2000). While this theory is generally accepted, studies have shown that the increased incidence of asthma, rhinitis, or Neurodermitis does not completely account for decreased microbial exposure (**Mallol, 2008**; **Brooks et al., 2013**; **Kramer et al., 2013**). Asthma is a widespread illness globally and affects individuals of all ages, This condition usually occurs in infancy and is characterized by variable symptoms of wheeze, dyspnea, and chest tightness caused by air flow obstruction (fully reversible) (**GINA, 2015**; **Bisgaard & Bonnelykke, 2010**). Conventional and Real time- PCR, the polymerase chain reaction (PCR) is a DNA replication test tube system that enables several million folds of a "target" DNA sequence to be selectively amplified in just a few hours. A predetermined fragment of DNA (the target, which can be from 100 to 1000 bp long, for example) is amplified by the PCR.

2. Materials and Methods

Samples

A total of (312) patients (149 males and 163 females) of various age groups were included in this Case–control study. The patient was examined, and diagnosed as asthma under supervision of the Physician. the study was carried out during a period from July 2018 to January 2020.

Control group

A total of (204) healthy individual (81 males and 123 females) with out any features of asthma or any allergies to be compared with asthmatic patient in genetic and immunological studies.

Molecular Study

DNA extraction from the blood samples

The genomic DNA from the whole blood for 100 patients was extracted according to DNA has been extracted from the blood by using gSYNC™ DNA Extraction Kit Quick protocol by Gene aid company (S. Koria).

Protocol

1) Up to 200µl of the whole blood were transferred to a 1.5 micro centrifuge tubes.
2) Proteinase k (20µl) was added and mixed by pipetting, then the incubate at 60°C for 5 minutes.
3) A 200µl of GSB lysis buffer was added then mixed by shaking vigorously, and incubated at 60°C for 5 minutes, tubes should be inverted every 2 minutes
4) absolute ethanol (200µl) was added to the sample lysate and mixed immediately by shaking vigorously for 10 seconds.
5) GS column was placed in a 2 ml collection tube and all the mixture was transferred to the GS column, centrifuged at 14000 x g for 1 minute, the 2ml collection tube containing the flow- through was discarded then the GS column was transferred to a new 2 ml collection tube.
6) 400µl of W1buffer were added to the GS column, centrifuged at 14000 x g for 30 seconds then the flow – through was discarded.
7) The GS column was placed back in the 2 ml collection tube.

8) wash buffer (600 µl) was then added to the GS column centrifuged at 14000 x g for 30 second than discarded the flow-through.
9) The GS column was placed back in the 2 ml collection tube, centrifuged again for 3 minutes at 14000 x g to let the column matrix dry.
10) The dried GS column was transferred to a clean 1.5 ml micro centrifuge tube.
11) Pre-heated elution buffer (100µl) was added in to the center of the column matrix, allowed to stand for at least 3 minutes to let elution buffer be completely absorbed, and then it was centrifuged at 14000 x g for 30 second to elute purified DNA.

Agarose Gel Electrophoresis
DNA was visualized by 1% agarose gel electrophoresis stained with ethidium bromide according to (Sambrook et al., 1989).

Reagents
1- TBE buffer (0.5x).2-Agarose.
3-Ethedium bromide (10mg/ml).4- DNA marker.
5- Bromophenol blue in 1% glycerol.

Protocol
Preparation of agarose gel was performed according to the method of Brody and Kern, (2004). in three steps:

A- Preparation of Agarose Gel
1) Agarose (1.5gm) was dissolved in 100ml Tris borate buffer (0.5x).
2) Agarose was heated in microwave until bubbles appeared for one minute.
3) Ethedium bromide (0.5µl) was added and mixed with the agarose solution.

B- Casting of the horizontal agarose gel
1) Both edges of the gel tray were sealed and the comb was positioned at one end of the tray.
2) Gel was poured into the tray, waited to harden.
3) Combs were removed gently and the gel tray was replaced in electrophoresis chamber.
4) TBE buffer was added to the chamber until it reached 5mm over the surface of the gel.

C- Loading and running DNA in agarose Gel
The reaction volumes were mixed on a piece of parafilm as follows:
1) DNA (9µl) was mixed with bromophenol blue in the ratio of 3: 1 and loaded in the wells of the 1% agarose gel.
2) Ladder DNA (5µl) was added to its place in the gel (only used with amplified DNA and not with whole DNA)
3) The cathodes were connected to the well side of the unit and the anode to the other side.
4) The gel was run at 60V (10 mA) until bromophenol blue was near the end of the gel.
5) Gel was removed and visualized bands under UV light (300 nm).

NOTE: Gel electrophoresis was done for whole DNA for all samples.

Polymerase Chain Reaction (PCR)
The PCR amplification of IL 4, IL17 were done in Gene X Privte lab. (Al- Muthna, Iraq).

Preparation of PCR primers
The primers are prepared depending on the manufacturing instruction by dissolving the lyophilized primers with TE (Tris-EDTA) buffer to make stock solution of concentration of 100 p mole/Ml, On spinning down and stay overnight at 4°C, primers working solution were prepared by diluting the stock solution with TE buffer to get final working solution (10 p mole/Ml) for each primer.

The RT PCR amplification. protocol
1) The volume of each component needed was calculated:
2) The Preparation of reagents:
   - The primer was mixed completely Thaw, then vortexed for 3 to 5 seconds and centrifuged briefly before openining the tube.
   - The PCR Reaction Mix was swirled gently before use. with not overtaxing.
3) The required volumes of components was Pipetted into an appropriately sized polypropylene tube.
4) The PCR mix was vortexed for 3 to 5 seconds, then briefly centrifuged.
5) The PCR mix (25 µl) was dispensed into each reaction well.
6) The sample, standard (2 µL) was added to the appropriate wells. For plate setup examples,
7) The reaction plate was sealed with the Optical Adhesive Cover.
8) The plate was centrifuged at 3000 rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles.
9) The compression pad was placed over the Optical Adhesive Cover with the gray side down, the brown side up, and the holes positioned directly over the reaction wells.
10) The reactions were running on the appropriate Applied Biosystems instrument.

The PCR amplification protocol
The PCR reactions on an Applied Biosystems Gene Amp ® veritiy, were performed. Amplification reactions of PCR product lines were tested for approximately one hour on an agarose gel of 1.5 %, using electrophoresis at 60 volts. Mixing 25 mL of 1X TBE (Tris borate-EDTA) and 0.5 g of a agarose (Bio basic, USA) in a flask and heating the mixture to the boiling temperature primed the gels. The flask was allowed to cool down to 45° C and coated with ethidium bromide once the agarose powder had completely dissolved. A maximum of 5 µL of the PCR product and 2 µL of the loading dye was combined and applied to each well. The first well contained a 100 bp ladder (Invitrogen, UK) against which the PCR products were measured, while the second well contained a negative control (with PCR combination but without DNA). The rest of the wells held the amplified DNA. The PCR products were viewed under ultraviolet light an hour later.

Table 1: Quantity of reaction mix for quantification of DNA

Component	Volume Per Reaction (µL)
Primer Mix	10.5 µl
PCR Reaction Mix	12.5 µl
Genomic DNA	2 µl
Total reaction	25 µl

Table 2: The qPCR amplification protocol

No. of stage	step	temp	Time	No. of cycle
1	Initial Denaturation	94˚C	5 min	1
2	Denaturation	94˚C	30sec	40
	Annealing	55˚C	52sec	
	Extintion	72˚C	60 sec	

Table 3: Quantities of PCR reaction mix

Item	Quantity
Master mix	10 µl
Forward Primer	1µl
Reveres primer	1µl
Genomic DNA	3µl
D. D. W	5µl
Total reaction	20 µl

Table 4: The PCR amplification protocol

No. of Stage	step	temp	Time	No. of cycle
1	Initial Denaturation	95˚C	5 min	1
2	Denaturation	94˚C	60sec	35
	Annealing	56˚C	30sec	
	Extintion	72˚C	60 sec	
3	Final Extintion	72˚C	5 min	1

Table 5: Primers

Primer		Primer sequence	MW	Reference
IL 4	F	GGATGTGTTTAGGTTCCATTCA	710	Correlation between IL-4 and IL-13gene polymorphisms and asthma in Uygur children in Xinjiang (JI-Hong Zhang, 2018)
	R	CCTCCTGGGGAAAGATAGAGTAA		
IL17A	F	CAGAAGACCTACATGTTACT	344	Single nucleotides polymorphism of IL 17 gene are associated with asthma (Jin Du, 2010)
	R	GTAGCGCTATCGTCTCTCT		

Statistical analysis

Statistical analysis is done by using statistical package for social sciences (SPSS) software version 11, the chi square test, univariate and multivariate logistic regression analysis, the ANoVA analysis were applied for correlation between each study parameter, and the difference between two proportion by T- tests were used to assess the significance of difference between groups, P-Value less than 0. 05 was considered as Statistically significance (S). P-value< 0. 01 as highly significant (HS). and P-value <0. 001 as extremely significant (ES).

3. Results

Conventional PCR

1- The DNA of the sample shown in figure (1).

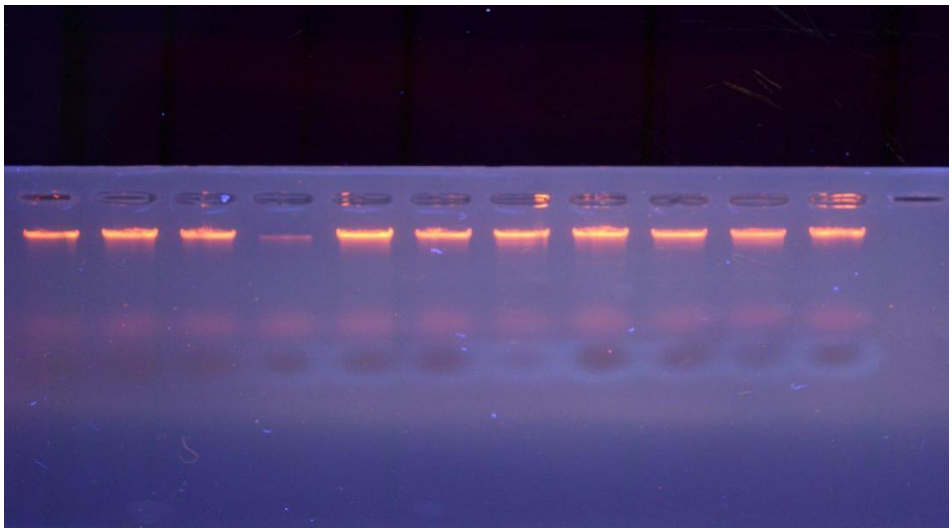

Figure 1: Ethidium Bromide stained agarose gel electrophoresis appearance that displays Genomic DNA that was extracted from (blood)

2-MW for each gene

IL-4 present in 85% of all student samples with MW of IL-4 gene aqual to 710 bp while IL-17 present 75% of all students samples with MW of IL-17 gene aqual to 344 bp.

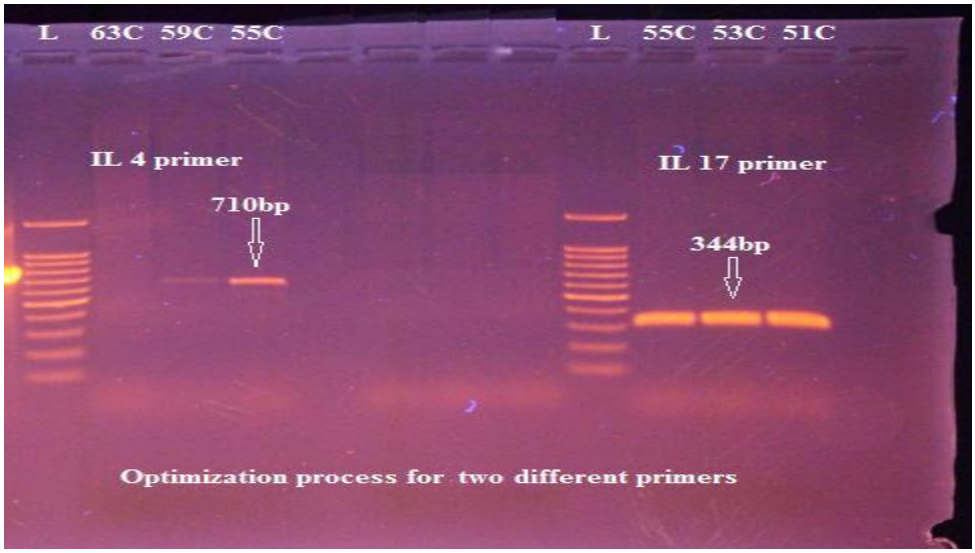

Figure 2: Gel electrophoresis for optimization process with 8 different temperatures for PCR product of 2 primers (IL4 and IL17 primers) show (710bp and 344bp) respectively. (Agarose 1%, 10min. at 100 voltage and then lowered to 70 volts, 60min.). Visualized under U. V light after staining with Ethidium bromide. Lane L: DNA ladder (1500-100) bp

RT-qPCR

The amplification of IL-4and IL-17 gene were carried by using Real time polymerase chain reaction techniques and the results illustrate in Figures 3-121 and 3-122.

The present study show that Amplification plots of genes for IL-4and IL-17 were started between cycle 17-20 and 16-23 respectively.

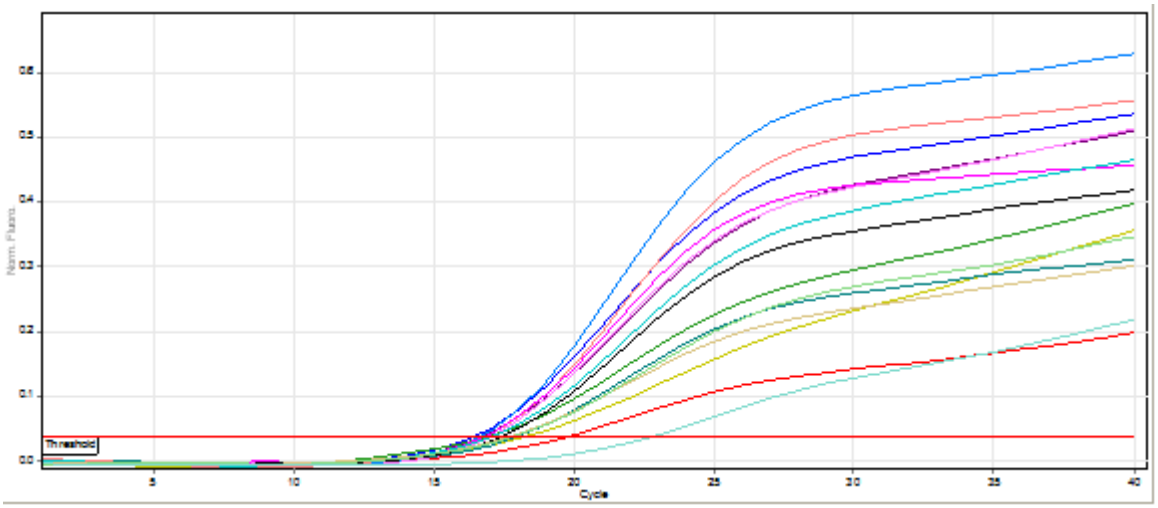

Figure 3: RT PCR IL-4

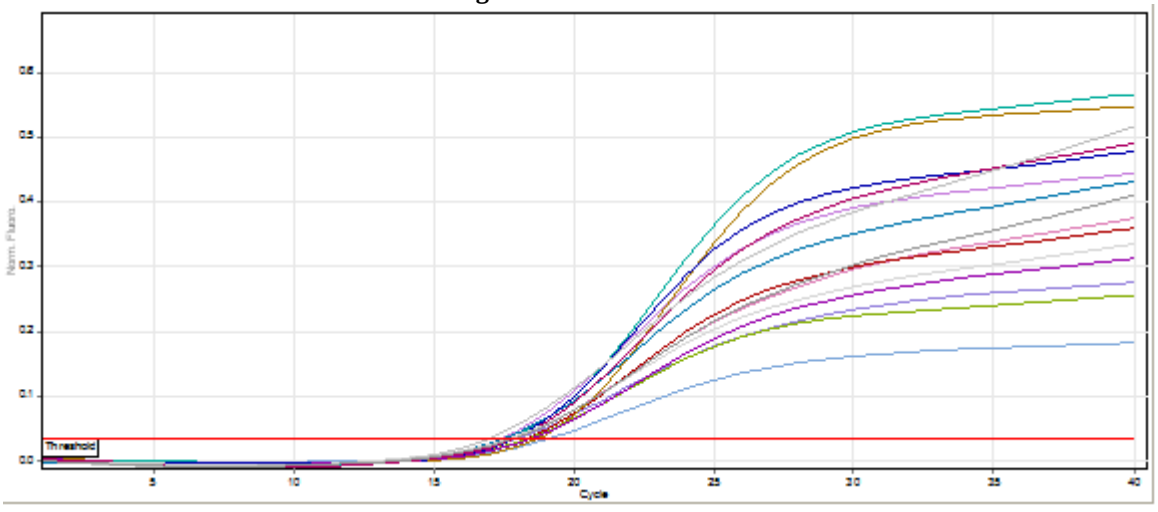

Figure 4: RT PCR IL-17

4. Discussion

Based on the implementation of PCR, DNA-based techniques have been further simplified. In trying to use PCR for genome analysis and specification purposes, different techniques have been adopted (**Fairbrother, et al., 1998).** The molecular expression study include extracting DNA from blood of Asthmatic patients, Asthmatic patients and Control group by using specific primers for conventional PCR and Real Time PCR, the amplification of all extracted DNA from blood samples was preform and confirm by using electrophoresis with (100/30min), the result of this estimation revealed that the amplified DNA (PCR product) was 710 bp for IL-4 (50voltage for 1hour) and the presence of IL-4 was 85% from total samples, (PCR product) was 344 bp for IL-17 on agarose gel (1%) The presence of IL-17 was 70%

RT PCR IL-4 show peak between 84.5 – 85.2 and IL-17 peak between 86.5-87.5, Results of the present study indicate that the IL-4, IL-17 associated with risk of Asthma, the association between SNPs and predisposition to certain diseases has been shown to depend on several factors among which ethnic background, and/or prior immune deficiencies **(Figueroa, *et al*., 2012),** and agree with **Wang and Wills (2011)**

Refrences

[1] **Alsaimary, Ihsan Edan A. Alsaimary, Hussein N. AlDhaheri, Murtadha M. ALMusafer.** Assessment of Immunomolecular expression and prognostic role of TLR7. Journal of Medical Research and Health Sciences among patients with Prostatitis.3 (11), 1105-1109 (2020) DOI: https: //doi.org/10.15520/jmrhs.v3i11.278

[2] **ALMusafer, Murtadha M. Hussein N. AlDhaheri Dr. Ihsan Edan A. AlSaimary.** Clinical study of patients with prostatitis in Basrah and Missan governments: a case –control study. Journal of Medical Research and Health Sciences.3 (11), 1110-1115 (2020). DOI: ttps: //doi.org/10.15520/jmrhs.v3i11.279

[3] **AlSaimary, Ihsan E., Hussein N. AlDhaheri, Murtadha M. ALMusafer. Molecular.** Gene Expression of Toll-Like Receptors 4 & 10 in Cellular Subsets of Human Peripheral Blood among Patients with Prostatitis: Conventional, Real Time Pcr and DNA Sequencing Techniques. International Journal of Medical Science and Clinical Invention 7 (11): 5095-5102 2020. DOI: 10.18535/ijmsci/v7i11.06

[4] **AlDhaheri, Hussein N., Ihsan E. AlSaimary and Murtadha M. ALMusafer.** PREVALENCE, INCIDENCE ESTIMATION, RISK FACTORS OF PROSTATITIS IN SOUTHERN IRAQ: CASE-CONTROL OBSERVATIONAL STUDY. EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH, 2020, 7 (12), 47-55

[5] **AlSaimary, Ihsan E Hussein N AlDhaheri and Murtadha M ALMusafer.** Immunomolecular Expression of TLR1 and TLR2 Genes Related With Prostatitis. Journal of Biotechnology & Bioinformatics Research. Volume 2 (4): 1-4

[6] **Alyasin, S., Karimi, M. H., Amin, R., Babaei, M. and Darougar, S. (2013).** Interleukin-17 gene expression and serum levels in children with severe asthma. Iranian journal of immunology, 10 (3): 177-185.

[7] **Amelink, M.; De Nijs, S. B.; De Groot, J. C.; Van Tilburg, P. M.; Van Spiegel, P. I.; Krouwels, F. H.; Lutter, R.; Zwinderman, A. H.; Weersink, E. J.; Ten Brinke, A.; Sterk, P. J. and Bel, E. H. (2013).** Three phenotypes of adult-onset asthma Allergy: 68 (5): 674-680.

[8] **Anand, K. S. S.; Ravi, G. N. and Narasimhaswamy, K. N. (2016).** Study of pulmonary function test in allergic rhinitis patients of Sullia, Dakshina Kannada. Journal of Evolution of Medical and Dental Sciences.; 5 (79): 5867-5870..

[9] **Wang, Y. H., Voo, K. S., Liu, B., Chen, C. Y., Uygungil, B., Spoede, W., Bernstein, J. A., Huston, D. P. and Liu, Y. J. (2010).** A novel subset of CD4+ TH2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. Journal of Experimental Medicine, 207 (11): 2479-2491.

[10] **Ward, C.; Pais, M.; Bish, R.; Reid, D.; Feltis, B.; Johns, D. and Walters, E. H. (2002).** Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. Thorax; 57 (4): 309-316.

[11] **Waser, M.; Michels, K. B.; Bieli, C.; Flöistrup, H.; Pershagen, G.; Von Mutius, E.; Ege, M.; Riedler, J.; Schram-Bijkerk, D. and Brunekreef, B. (2007).** Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. Clinical & Experimental Allergy.37 (5): 661–670.

[12] **Wasserman, R. L.; Factor, J. M.; Baker, J. W.; Mansfield, L. E.; Katz, Y.; Hague, A. R.; Paul, M. M.; Sugerman, R. W.; Lee, J. O. and Lester, M. R. (2014).** Oral immunotherapy for peanut allergy: multipractice

[13] **Savio, J.; Ramachandran, P.; Jairaj, V.; Devaraj, U. and D'Souza, G. (2019).** A cross-sectional study of skin prick test to Aspergillus fumigatus antigen in asthmatic patients seen at a tertiary healthcare center. Indian Journal of Allergy, Asthma and Immunology.; 33 (1): 19.

[14] **Schatz, M. and Rosenwasser, L. (2014).** The allergic asthma phenotype. The Journal of Allergy and Clinical Immunology: In Practice.; 2 (6): 645-648.

[15] **Schaub, B.; Lauener, R. andVon Mutius, E. (2006).** The many faces of the hygiene hypothesis. J. Allergy Clin. Immunol.; 117 (5): 969, 977.

[16] **Schiffman, George. (2009**). "Chronic obstructive pulmonary disease". Med. Net. Science; 282: 2258–2261.