

Research Article

Some Immunological Aspects of Iraqi Influenza A Virus Infections

Raghad H. Al-Azzawi

Department of Biology, College of Science, University of Baghdad, IRAQ

*Correspondent Author Email: raghadh2004@yahoo.com

Article Info

Received
22 Nov. 2016

Accepted
12 Jun. 2017

Abstract

In the last years, the world has been facing pandemic influenza which caused by influenza A virus. In this study detection of influenza A virus infection in Iraqi patients was done by r RT-PCR. Three hundred eighty (380) clinical respiratory secretions samples that were collected between December and April 2013, from different hospitals and they were sent to Central Public Health Laboratory (CPHL)/National Influenza center/ Baghdad /Iraq. Among these samples only 35 samples (22 Males and 13 females all of them were adults) gave positive results (both TS (throat swabs) and NPS (nasopharyngeal swab)) for influenza A virus (9.2%). Interleukin-6 (IL-6) and IL-1 α are pleiotropic cytokines implicated in the pathogenesis of local inflammation during viral upper respiratory infections. Cytokines levels in both NPS and TS were determined. The result appeared that there was a significant difference between IL-6 and IL-1 α , but there was no significant difference between IL-6 and IL-1 α in Ts and NSP. For the first time we try to detect C-reactive protein (CRP) in nasopharyngeal swab (NPS) and throat swabs (TS) and compared with serum, also to prove that this protein may be secreted in fluids other than serum. The result showed that **64.3% of both NPS and TS** were positive for CRP. This may be due to that CRP in response to microbial infection, tissue injury, and immunomodulatory stimuli is synthesized and released by various cells.

Keywords: influenza A virus; CRP; IL-6; IL-1 alpha.

الخلاصة

بدء العالم في السنوات الاخيرة يواجه مشكلة وباء الانفلونزا المتسبب عن فيروس الانفلونزا نمط أ. في هذه الدراسة تم تشخيص اصابات الانفلونزا باستخدام تقنية تفاعل البلمرة المتسلسل بطريقة النسخ الراجع في الوقت الحقيقي (rRT-PCR). من بين 380 عينة من افرازات الجهاز التنفسي المأخوذة من مختلف المستشفيات للفترة مابين كانون الاول ونيسان 2013 والتي ارسلت الى مختبر الصحة المركزي/يغداد لتأكيد التشخيص. فقط 35 عينة (منهم 22 ذكر و 13 انثى بالغين) اعطت نتيجة موجبة (لعينتي مسحات الحنجرة والافرازات البلعومية الانفية) للاصابة بفيروس الانفلونزا نمط أ وبنسبة 9.2%. تم قياس مستوى الانترلوكين-6 والانترلوكين-1 الفا (وهما من الساييتوكينات المتعددة المظاهر التي لها دور في الالتهابات الموضعية اثناء الاصابات الفيروسية للجهاز التنفسي العلوي) في عينات مسحات الحنجرة والافرازات البلعومية الانفية واطهرت النتائج بأنه لا توجد فروقات معنوية بين IL-6 و IL-1 α في هذه العينات. وللمرة الاولى نحاول الكشف عن افراز البروتين C الفعّال CRP في مسحات الحنجرة والافرازات البلعومية الانفية ومقارنته مع تركيزه في مصل المرضى وكذلك لاثبات بأن هذا البروتين يمكن ان يفرز في سوائل جسمية اخرى غير المصل. و اظهرت النتائج بأن 64.3% من عينات مسحات الحنجرة والافرازات البلعومية الانفية ايجابية للبروتين CRP. وهذا من الممكن ان يرجع هذا الى ان البروتين CRP يصنع ويفرز من قبل مختلف الخلايا كاستجابة للاصابات الميكروبية, جروح الانسجة, والتحفيز المناعي.

Introduction

Influenza is an infectious disease, commonly known as "the flu", caused by many reasons one of them is the influenza virus [1]. The most

common symptoms, which could be mild to severe, include: a high fever, sore throat, runny nose, muscle pains, coughing, headache, and feeling tired [2]. These symptoms typically begin



two days after exposure to the virus and most last less than a week. The cough, however, may last for more than two weeks [1]. Viral pneumonia, secondary bacterial pneumonia, sinus infections, and worsening of previous health problems such as asthma or heart failure are complications of influenza [1] [2]. Influenza caused a yearly outbreak that spreads around the world, resulting in about three to five million cases of severe illness and about 250,000 to 500,000 deaths [1].

Influenza A virus is the causative agent of both epidemic and pandemic flu, which are envelope, single stranded and negative-sense RNA viruses [3]. The genome of influenza A virus segmented into eight RNA molecule, each fold into rod shaped double-helical ribonucleoprotein complex (RNP) [4].

The natural hosts for a large variety of influenza A are wild aquatic birds. Occasionally, viruses could be transferred to other species and might then cause devastating outbreaks in domestic poultry or cause human influenza pandemics [3]. The type A viruses of (A, B and C) are the most virulent human pathogens among the three influenza types and cause the most severe Disease dependent on the antibody response to these viruses, the influenza A virus can be subdivided into different serotypes [5].

Upregulation of inflammatory cytokines, such as the TNF- α , IL-1 β , IL-6, and IL-10, and a cytokine-mediated inflammatory response have also been documented as responsible of severity of viral lung infections [6] [7].

Recent studies demonstrated that influenza virus A elicits an acute inflammatory response characterized by the production of pro-inflammatory cytokines, such as IL-33 and IL-6, in infected lungs, suggesting a key role for these interleukins in the lung inflammation and pathogenesis of respiratory epithelial cell damage [8] [9].

C - reactive protein is a special type of protein found in blood plasma is synthesized by the liver in response to factors released by macrophages and fat cells (adipocytes) [10]. The circulating

concentration of which rises dramatically in a cytokine-mediated response to most forms of tissue injury, infection and inflammation, serum CRP values are widely measured in clinical practice as an objective index of disease activity [11]. Expression of CRP is regulated mainly at the transcriptional level with inter leukin-6 being the principle inducer of the gene during the acute phase [10].

The aim of this study was to detect the influenza A virus infection in Iraqi patients by r RT-PCR and evaluate the interleukin IL-1 α and IL-6. Also in order to investigate the expression of CRP in nasopharyngeal wash and throat swabs and compared them, for the first time, with the concentration of CRP in serum, this study was done.

Materials and Methodology

Collection of samples:

This study was performed using (384) clinical samples of nasopharyngeal secretions (NPS) and throat swabs (TS) and screened for the presence of Influenza A virus. The samples were collected from different hospitals and they were sent to Central Public Health Laboratory (CPHL)/National Influenza center/ Baghdad /Iraq between December and April 2013 from patients of different sexes and different cities with respiratory distress symptoms. The samples were collected in 5 ml of transport medium (phosphate-buffered saline containing 10% glycerol, 1 mg/ml gentamicin together with 8 IU/ml of penicillin, 8 μ g/ml of streptomycin, 0.02 IU/ml of amphotericin B (Invitrogen, Carlsbad, CA) and stored at 4°C. Also, 5 ml blood sample were collected. The serum was separated and stored at -20°C taking precaution by avoiding repeated freezing and thawing of the samples by saving them separately for CRP test. Also, 20 samples were collected from healthy persons.

Detection of Influenza A virus infection by real-time RT-PCR(rRT-PCR):

Extraction of RNA:

This was done using QIA prep Spin RNA Miniprep Kit and the procedure was done according to the kit instructions [12]. Specific primers for influenza A , (Sigma,USA) were used the primers,InfA forward: GAC CRA TCC TGT CAC CTC TGA C, InfA reverse:AGG GCA TTY TGG ACA AAK CGT CTA, InfA probe TGC AGT CCT CGC TCA CTG GGC ACG.

Detection the Infection

Quantitative analysis was performed on a StepOne Plus Taqman Real Time PCR for detection the infection with influenza virus A using Directigen Flu A kits(Becton Dickinson, Cockeysville, Md.) and the test was done according to the instructions.

Determination of Cytokines:

Cytokines levels in both NPS and TS were determined. The cytokines that used in this assay were IL-6, IL-10, and IL-1 α , which were measured by means of enzyme-linked immunosorbent assay, as recommended by the manufacturer, using ELISA kits (Boster biological technology Co.). Cytokines were measured by using a solid phase sandwich ELISA.

Determination of C - reactive protein (CRP)

The serum and (NPS and TS) levels of CRP were measured by using enzyme immunoassay kit (Monobind Inc., CA, USA). In briefly, samples were added to a streptavidin coated wells, after that biotinylated monoclonal and enzyme labeled antibodies were added. A complex formed by the reaction between the

CRP antibodies and native CRP. The enzyme CRP antibody bound conjugate is separated from unbound one, after incubation and washing. Finally, the substrate is reacted with enzyme present on the wells to produce color.By using microplate reader the absorbance was read at 450 nm.

Statistical test:

In order to evaluate the variables in this study, T-test has been used to test the significant between each tow variables at P-value ($P \leq 0.05$) using Excel application and SPSS program to find the results.

Result and Discussion

Distribution of infection

Among 380 clinical respiratory secretions, 35 samples gave positive results (both TS and NPS) for influenza A virus (9.2%) (22 Males and 13 females all of them were adults).

Other study showed that 11 out of 598 laboratory-confirmed influenza cases caused by influenza A(H1N1)pdm09 have died, while 587 patients recovering completely in Iraq [13].

While another study of a total of 1059 people, registered as suspected cases of pandemic influenza An (H1N1) virus in Kurdistan province, that included in the study, the disease was confirmed in 157 cases (14.8%) [14].

Cytokines levels

The results of the comparison study in IL-6 and IL-1 α between both throat swabs (TS) and nasopharyngeal secretions (NPS) (table 1) showed that there was no significant difference, but there was a significant difference between IL-6 and IL-1 α in TS samples.

Table 1: The comparisons between the study parameters.

Parameters	Comparisons	Test of variances	t-cal.	t-table	Significant at $p \leq 0.05$
IL-6 Pg/ml(control= 6.904)	TS versus NPS	Equal	-0.855	-2.025	Non-Significant
IL-1 α (control=	TS versus NPS	Equal	-0.0004	-2.025	Non-Significant



16.238)							
TS	IL-6 Pg/ml	versus	IL-1C	Unequal	-3.1243	-2.093	Significant
NPS	IL-6 Pg/ml	versus	IL-1 α	Equal	-1.7857	-2.025	Non-Significant
Male TS	IL-6 Pg/ml	versus	IL-1 α	Unequal	-2.8390	-2.228	Significant
Female TS	IL-6 Pg/ml	versus	IL-1 α	Unequal	-1.4546	-2.120	Non-Significant
TS	Male IL-6 Pg/ml	versus	Female IL-1 α	Unequal	-3.4135	-2.101	Significant
TS	Male IL-1 α	versus	Female IL-6	Equal	1.2881	2.101	Non-Significant

(TS) Throat swabs and (NPS) nasopharyngeal secretions

A study of de la Tabla *et.al.*,2010 about detection of influenza they used combination of nose and throat swabs versus NP aspirates. They founded that the performance of nasal swabs alone was not evaluated, but the combined swabs had a higher diagnostic yield [15]. Other study using NP aspirates reported that 88% sensitivity for the detection of influenza A, and sensitivity of 84% with the nasal swab when tested by PCR; the results showed the difference in sensitivities was not statistically significant ($P=0.72$) [16]. A study of comparison between the sensitivities of two specimen collection methods by using two diagnostic methods for the diagnosis of influenza noticed the nasal swab was less sensitive than the NP swab, irrespective of a diagnostic test, but the difference in sensitivities was not significant between sampling methods [17].

Lambert and colleagues compared combination of nose-throat swabs with NP aspirates in a large pediatric population. The results appeared the sensitivities of 100% for influenza B and the nose-throat swab were 92% for the detection of influenza A and [18].

The relationships between IL-6 and IL-1 α in Ts and NPS were demonstrated in figure (1). There were no significant differences seen between TS and NPS in both IL-6 and IL-1 α .

When compare the results between males and females in TS we founded that there were

significant difference in IL-6 and IL-1 α in males, while in females, no significant differences were be seen (Figure 2). But there were significant differences between male IL-6 and female IL-1 α in TS samples (Figure3).

CRP in serum and (in both samples of nasal wash and throat swabs) was tested for the first time in this study as trying to prove that this protein may be secreted in fluids other than serum. All fourteen (14 out of 35 available) serum samples were showed a positive result for CRP in acute influenza A virus patients. While only nine (9) nasal wash and throat swabs were showed a positive result for this test (Table 2).

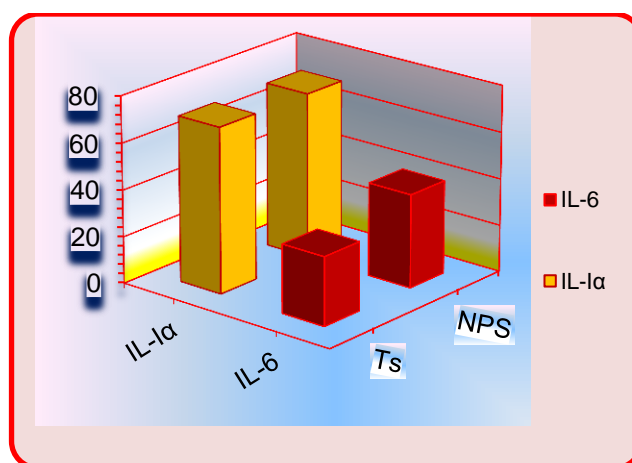


Figure 1: The relationship between IL-6 and IL-1 α in both TS and NPS. (TS) Throat swabs and (NPS) nasopharyngeal secretions.

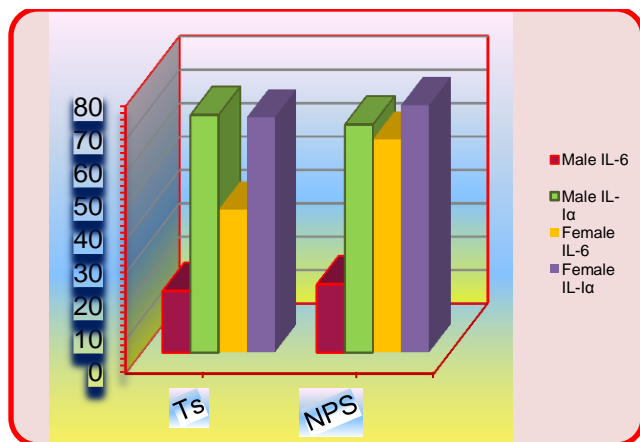


Figure 2: Comparison between TS (throat swab) and NPS (nasopharyngeal secretion) in male and female IL-6 and IL-1 α .

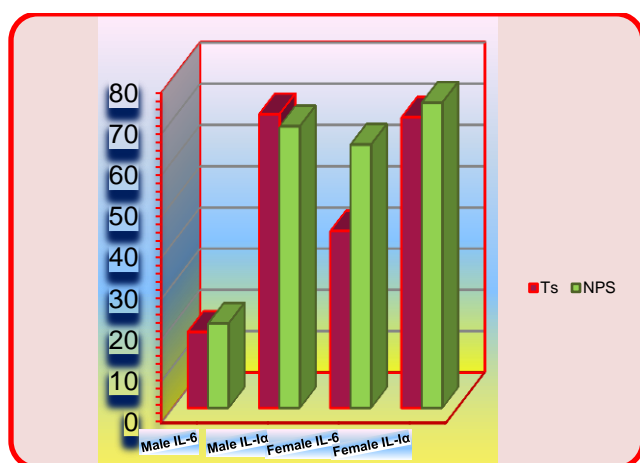


Figure 3: Comparison between male and female IL-6 and IL-1 α in TS (throat swab) and NPS (nasopharyngeal secretion).

CRP in response to microbial infection, tissue injury, and immunomodulatory stimuli, is synthesized and released by various cells. The results have demonstrated that vascular and organ-specific cells *In vitro* can produce CRP in response to inflammatory stimuli [19] [20]. The ability of peripheral blood mononuclear cells (PBMC) to express and release CRP, despite elaborate evidence on the generation of CRP by different cell types, has not yet been demonstrated. But some studies have in PBMC [21] [22]. This may be attributed to phagocytosis rather than to constitutive expression [23]. Maier

et al (2005) reported that the cellular effectors of the innate immune response system is PBMC and accumulate in compartments where CRP is highly concentrated, i.e. in arteriosclerotic plaques [24]. Therefore this results lead to hypothesize that CRP may also be generated and released by PBMC.

This result coincides with other studies that found increasing in cellular CRP expression to TNF- α , LPS, or other cytokines [22] [25]. However, importantly notice that the alveolar macrophages may respond differentially to CRP [26] and therefore divergent effects of cytokine stimulation, may occur, depending on the target cells.

Bedaiwy and Falcone (2003) mentioned that the major source of cytokines is the macrophages. Cytokines that are originated in bone marrow circulate as monocytes and move to different body cavities, [27] and CRP activation in peripheral blood mononuclear cells can also be stimulated by cytokines that are released abundantly during the inflammatory process [22].

Table (2): The result of CRP in acute influenza A virus patients by enzyme immunoassay kit.

Sample Number	Throat swab	Nasal wash	serum	Sex
1	+	+	+	male
2	-	-	+	female
3	+	+	+	male
4	+	+	+	female
5	+	-	+	female
6	-	-	+	male
7	+	+	+	male
8	+	+	+	male
9	-	+	+	male
10	+	-	+	female
11	+	+	+	male
12	-	-	+	male
13	+	+	+	female
14	-	+	+	female
%	64.3	64.3	100	

Conclusions

CRP can be secreted and detected in fluids other than serum like in TS (throat swab) and NPS (nasopharyngeal secretion). Also there were differences in cytokines concentration in these samples and in both males and females Iraqi influenza virus patient.

References

- [1] World health organization Influenza (Seasonal) Fact sheet NO 211, March 2014.
- [2] World health organization "Key Facts about Influenza (Flu) and Flu Vaccine". September 9, 2014. Retrieved 26 November 2014.
- [3] Klenk, Hans-Dieter; Matrosovich, Mikhail; Stech, Jürgen. (2008). "Avian Influenza: Molecular Mechanisms of Pathogenesis and Host Range". In Mettenleiter, Thomas C.; Sobrino, Francisco. *Animal Viruses: Molecular Biology*. Caister Academic Press. ISBN 978-1-904455-22-6.
- [4] Wenjie Z; John O; Vikram V; and Yizhi J T. (2013). The Crystal Structure and RNA-Binding of an Orthomyxovirus Nucleoprotein. *PLoS Pathog.* 9(9): e1003624.
- [5] Stephanie A. Irving, Mary F. Vandermause, David K. Shay, and Edward A. (2012). *Belongia*. Comparison of Nasal and Nasopharyngeal Swabs for Influenza Detection in Adults. *Clinical Medicine and Research*. 10 (4): 215-218.
- [6] Estella, A. (2011). Cytokine levels in bronchoalveolar lavage and serum in 3 patients with 2009 influenza A(H1N1)v severe pneumonia. *Journal of Infection in Developing Countries*. 5, (7): 540–543.
- [7] Antonio, C, Silvia P., Giovanni B., Pietro F., Valerio R., Domenico C., and Riccardo R. (2013). IL-1 β and IL-6 Upregulation in Children with H1N1 Influenza Virus Infection. *Mediators of Inflammation*.
- [8] R. Le Goffic, M. I. Arshad, M. Rauch. (2011). "Infection with influenza virus induces IL-33 in murine lungs," *The American Journal of Respiratory Cell and Molecular Biology*. 45(6):1125–1132.
- [9] A. G. Besnard, D. Togbe, N. Guillou, F. Erard, V. Quesniaux, and B. Ryffel. (2011) "IL-33-activated dendritic cells are critical for allergic airway inflammation," *European Journal of Immunology*. 41 (6): 1675–1686.
- [10] Russell RJ, Kerry PS, Stevens DJ, Steinhauer DA, Martin SR, Gamblin SJ, Skehel JJ. (2008). "Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion". *Proc. Natl. Acad. Sci. U.S.A.* 105 (46): 17736.
- [11] Nelson DL, Cox MM. *Lehninger's Principles of Biochemistry* (4th ed.). New York: WH Freeman. (2005).
- [12] Bustin, S. A and Mueller, R. (2005). Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clinical Science*. 109: 365–379.
- [13] WHO. (2013). H1N1 influenza cases reported in Iraq, 5 February.
- [14] Afrasiabian, S.; Mohsenpour, B.; Bagheri, K.H.; Barari, M.; Ghaderi, E.; Hashemi, R.; and Garibi, F. (2014). Epidemiological survey on pandemic influenza A (H1N1) virus infection in Kurdistan province, Islamic Republic of Iran, 2009. *EMHJ*. 20 (3).
- [15] de la Tabla VO, Masiá M, Antequera P, Martín C, Gazquez G, Buñuel F, Gutiérrez F. (2010). Comparison of combined nose-throat swabs with nasopharyngeal aspirates for detection of pandemic influenza A/H1N1 2009 virus by real-time reverse transcriptase PCR. *J Clin Microbiol.* 48:3492–3495.
- [16] Sung RY, Chan PK, Choi KC, Yeung AC, Li AM, Tang JW, Ip M, Tsen T, Nelson EA. (2008). Comparative study of nasopharyngeal aspirate and nasal swab specimens for diagnosis of acute viral respiratory infection. *J Clin Microbiol.* 46:3073–3076.

- [17] Stephanie A. I., Mary F. V.[‡], David K. S., and Edward A. B. (2012). Comparison of Nasal and Nasopharyngeal Swabs for Influenza Detection in Adults. *Clinical Medicine & Research*. 10 (4): 215-218.
- [18] Lambert SB, Whiley DM, O'Neill NT, Andrews EC, Canavan FM, Blethly C, Siebert DJ, Sloots TP, Nissen MD. (2008). Comparing nose-throat swabs and nasopharyngeal aspirates collected from children with symptoms for respiratory virus identification using real-time polymerase chain reaction. *Pediatrics*. 122:e615–e620.
- [19] Calabro P, Willerson JT, Yeh ET. (2003). Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells. *Circulation*. 108:1930–2.
- [20] Venugopal SK, Devaraj S, Jialal I. (2005). Macrophage conditioned medium induces the expression of C-reactive protein in human aortic endothelial cells: potential for paracrine/autocrine effects. *Am J Pathol*. 166:1265–71.
- [21] Maier W, Altwegg LA, Corti R. (2005). Inflammatory markers at the site of ruptured plaque in acute myocardial infarction: locally increased interleukin-6 and serum amyloid a but decreased C-reactive protein. *Circulation*. 111:1355–61.
- [22] Haider, D G ; Leuchten, N ; Schaller, G; Gouya , G ; Kolodjaschna , Kolodjaschna; Schmetterer, L ; Kapiotis , S; Wolzt, M. (2006). C-reactive protein is expressed and secreted by peripheral blood mononuclear cells. *Clin Exp Immunol*. 146(3): 533–539.
- [23] Jabs WJ, Logering BA, Gerke P. (2003). The kidney as a second site of human C-reactive protein formation *in vivo*. *Eur J Immunol*. 33:152–61.
- [24] Moshage HJ, Roelofs HM, van Pelt JF. (1988). The effect of interleukin-1, interleukin-6 and its interrelationship on the synthesis of serum amyloid A and C-reactive protein in primary cultures of adult human hepatocytes. *Biochem Biophys Res Commun*. 155:112–17.
- [25] Pue CA, Mortensen RF, Marsh CB, Pope HA, Wewers MD. (1996). Acute phase levels of C-reactive protein enhance IL-1 β and IL-1ra production by human blood monocytes but inhibit IL-1 β and IL-1ra production by alveolar macrophages. *J Immunol*. 156:1594–600.
- [26] Bedaiwy MA, Falcone T. (2003). Peritoneal fluid environment in endometriosis. Clinicopathological implications. *Minerva Ginecol*. 55(4):333–45.