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Virological diagnosis of influenza viruses in zagazig university hospitals

Abstract

Influenza-like illness is a nonspecific respiratory illness characterized by fever, fatigue, cough, sore throat, headache and other symptoms that stop within a few days.

Aim of the work: Aim of this study was to expect influenza virus as a cause of influenza like illness depending on signs and symptoms and to compare rapid techniques and viral isolation for its diagnosis. Determine whether nasal or throat swabs are superior specimens to detect the seasonal influenza virus by measuring viral load in all specimens by real time RT-PCR. Compare qPCR assays with conventional RT-PCR to assess its potential application in routine surveillance and diagnosis.

Subjects, materials & methods: This study carried out in Medical Microbiology and Immunology department, Faculty of Medicine, Zagazig University. The study included 75 patients, 46 were males and 29 were females with ages ranging from 4 days to 65 years old (median=19). Nasopharyngeal swabs were obtained from all patients after 2 or 3 days of onset and were subjected to fluorescence detection of the viruses directly in the samples and a trial to isolate the viruses by cell culture on MDCK cells. One nasal and one throat swab were obtained from every patient and examined for influenza virus detection by both conventional and real time RT-PCR.

Results: Indirect fluorescence test performed directly on the nasopharyngeal samples revealed 23 positive cases out of 75. The cell culture revealed that 22 out of 75 cases were confirmed as influenza. In comparing Fluorescence versus the cell culture as the gold standard, its sensitivity =90.9%, specificity =94.3% and accuracy =93.3%. Positive predictive value =86.9% and Negative predictive value =96.1%. As regard Fluorescence before and after cell culture technique, it was statistically non significant. Age group (<2 years) was the most susceptible age group to influenza disease and there was male predominance (61.3%). Real time PCR detected more positive samples when compared to conventional RT-PCR. There is direct correlation between throat and nasal swabs real time RT-PCR and this is statistically significant. The mean, median, standard deviation and range of viral load by nasal swabs are higher than those of throat swabs denoting that nasal swabs are more useful diagnostic tools in diagnosis of influenza than throat swabs.

Keywords: Influenza, Immunofluorescence, RT-PCR

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Refat Sadeq, Heba Mohtady, Salwa Badrel Sabah, Maha Gohar, Fatma Amer, Shereen Atef, Rehab Rabia

Department of Microbiology, Zagazig University, Egypt

Correspondence: Refat Sadeq, Department of Microbiology, Faculty of Medicine, Zagazig University, Borg Agyad, Mashaya Mansoura, Egypt, Tel 002-01003737012, Email egyrefat@gmail.com

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Abbreviations: ILI, Influenza-Like Illness; ARI, Acute Respiratory Infection; SARI, Severe Acute Respiratory Infection; CDC, The Centers for Disease Control; PCR, Polymerase Chain Reaction; WHO, World Health Organization; RT-PCR, Reverse Transcription Polymerase Chain Reaction; RSV, Respiratory Syncytial Virus; HRV, Human Rhino Virus; HMPV, Human Meta Pneumovirus; HAdV, Human Adenovirus; HPIV, Human Para Influenza Viruses; HCoV, Human Corona Viruses; HBoV, Human Boca Virus; MDCK, Madin-Darby Canine Kidney; MEM-E, Minimum Essential Medium-Eagl's; FCS, Fetal Calf Serum; CPE, Cytopathic Effect

Introduction

Influenza-like illness (ILI), also known as acute respiratory infection (ARI) and flu-like syndrome, is a non specific respiratory illness or a medical diagnosis of possible influenza or other illness causing a set of common symptoms with severe acute respiratory infection (SARI). The Centers for Disease Control (CDC) defines a case of influenza like illness as an individual presenting with fever of 37.8°C or higher and one of the following symptoms (cough, sore throat, headache or muscle ache). In the absence of a physician's diagnosis or laboratory test results, individuals that present with influenza like illness (ILI) and meet the above criteria should be managed as cases of influenza.[1]

Appropriate treatment of patients depends on accurate diagnosis. Early diagnosis of influenza can reduce the inappropriate use of antibiotics and provide the option of using antiviral therapy. The accuracy of clinical diagnosis of influenza on the basis of symptoms alone is limited because symptoms from illness caused by other pathogens can overlap considerably with influenza.[2]

Diagnostic tests available for influenza include viral culture, antibody detection, antigen testing, polymerase chain reaction (PCR), and immunofluorescence assays. Sensitivity and specificity of any test for influenza might vary by the laboratory that performs the test, the type of test used, and the type of specimen tested.[1]

Influenza like illness is one of the leading causes of morbidity and mortality in infants and children, especially in developing countries. According to the World Health Organization (WHO), acute lower respiratory infections account for approximately 20% of all deaths among children less than 5 years and 70% of those deaths occur in Africa and Southeast Asia. The elderly and the immune compromised persons are also at risk to develop serious complications due to ILI.[3]

Several pathogens are implicated in ILI; most of viral infections especially in children are caused by Human respiratory syncytial virus (RSV),[4] Human rhinovirus (HRV) is responsible for the majority of common colds causing ILI,[5] Influenza virus causes infection that may be severe or fatal in the very young, the elderly, and those with underlying illnesses,[6] Human meta pneumovirus (HMPV)[7] and Human adenovirus (HAdV). In addition, infections caused by other viruses such as Human Para influenza viruses (HPIV), Human Coronaviruses HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1[8] and Human Boca virus (HBoV) have also joined the panel of virus responsible of ILI.[9]

Aim of this study was to expect influenza virus as a cause of influenza like illness depending on signs and symptoms and to compare rapid techniques and viral isolation for its diagnosis. Determine whether nasal or throat swabs are superior specimens to detect the seasonal influenza virus by measuring viral load in all specimens by real time RT-PCR. Compare qPCR assays with conventional RT-PCR to assess its potential application in routine surveillance and diagnosis.

Subjects materials and methods

This study included 75 patients, 46 were males and 29 were females with ages ranging from 4 days to 65 years old (median=19). This study follows regulations of ethical committee of Zagazig University. Nasopharyngeal swabs were collected on viral transport medium.[10] Procedure was done according to California Department of Public Health, 2009.[11]

Fluorescent staining was performed directly on samples using MONOFLUOTM KIT Influenza test (BIORAD-France). The MONOFLUOTM KIT Influenza test is intended for the detection of the influenza A and B virus in infected cells by means of indirect immunofluorescence technique using monoclonal antibodies specific to each of these viruses. These monoclonal antibodies bind to the antigen expressed in the cytoplasm of infected cells. The addition of mouse anti-IgG conjugate labeled with fluorescence makes the cells which have fixed the monoclonal antibody fluorescent. The cells that bind to the specific monoclonal antibodies directed against the virus proteins exhibit fluorescence, mainly cytoplasmic, with a granular or particulate appearance at the fluorescent microscope examination.

C- Cells

All samples were subjected to virus isolation by tissue culture technique using Madin-Darby Canine Kidney (MDCK) (Obtained from Holding Company for Biological products, Vaccines& Drugs (VACSERA-EVYVAC) (El Agoza-Egypt)) continuous cell line and D-cells: Minimum Essential Medium-Eagl's (MEM-E) (Obtained from Holding Company for Biological products, Vaccines& Drugs (VACSERA-EVYVAC) (El Agoza-Egypt).

Cells were grown in growth medium MEM-E, (7%) fetal calf serum (FCS), 100 IU/mL of penicillin, 100 µg/mL of streptomycin and 40 µg/mL of amphotericin-B.[12] After filtration cells were inoculated with samples and incubated at 37°C, 5% CO2 for 6 days. Cells were examined daily for cytopathic effect (CPE). Typing of influenza viruses were done by using immunofluorescent staining. Viral multiplication could be sufficient to be detected at this stage by immunofluorescence.[12] One nasal and one throat swab were obtained from every patient and examined for influenza virus detection by both conventional and real time RT-PCR. Copan universal transport medium (UTM-RT) system (COPAN-Italy). Each nasal and throat swabs sample was subjected to:

A. Extraction of viral nucleic acids from samples (by using AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit, Cat. No. AP-MN-BF-VNA-50, Axygen Biosciences)

B. Conventional reverse transcription PCR on RNA extracts

PCR reactions were all performed using TaqRT/PCR Master Mix Gold beads Kit (Cat No:-11020-96, 96 tubes, BIORON, Germany).

A forward primer, whose sequence was,

5′- ATGAGYCTTYTAACCGAGGTCGAAACG -3′.

A reverse primer, whose sequence was,

5′- TGGACAAANCGTCTACGCTGCAG-3′

These primers give a predicted product size of 244 bp.

A. Reverse transcription real time PCR on RNA extracts

i. Reverse transcription of RNA extracts was performed using RevertAid H Minus First Strand cDNA Synthesis Kit (Cat No.:- #K1631, Fermentas)

A forward GAPDH primer, 10 M

5' – CAAGGTCATCCATGACAACTTTG – 3'

A reverse GAPDH primer, 10 M

5' – GTCCACCACCCTGTTGCTGTAG 3'

ii. Real time PCR reaction on cDNA product of RNA extracts:

This was performed using Maxima SYBR Green qPCR Master Mix (2X) (cat NO. K0259, Lot 00069647, Fermentas).

A forward primer, whose sequence was,

5′- ATGAGYCTTYTAACCGAGGTCGAAACG -3′.

A reverse primer, whose sequence was,

5′- TGGACAAANCGTCTACGCTGCAG-3′

The thermal cycler was programmed according to conditions needed, the samples were placed in the cycler (Rotor-Gene Q 1.7.94) and program started.

Results

Table 1: Shows that patients were divided into 5 age groups which are (<2y), (2-15 y), (15-30 y), (30-45 y) and (45-65 y).

Table 1 Frequency table for the distribution of socio-demographic data among the studied patients with ILI

Item	No	%
Age (n=75)		
< 2	21	28
2-15	11	14.7
15-30	18	24
30-45	17	22.7
45-65	8	10.6
Sex (n=75)		
Female	29	38.7
Male	46	61.3

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Table 2: Shows the distribution of symptoms in patients with ILI during the period of this study.

Table 2 The distribution of symptoms in patients with influenza like illness

Item	No (n=75)	%
Fever	71	94.7
Sore Throat	31	41.3
Dry Cough	55	73.3
Rhinorrhra	43	57.3
Malaise	35	46.7
Chills	15	20
Headache	16	21.3
GIT	5	6.7

From this table we detected that the most common presenting symptom in patients with ILI was fever (94.7%), followed by cough (73.3%), then rhinorrhea (57.3%), while the least common symptom was GIT symptoms (6.7%). This study was carried out during four successive seasons (autumn 2010, winter 2010-2011, spring 2011 and summer 2011).

Table 3 Table 3 shows that most ILI patients were in winter (58.7%) followed by autumn (28%), and then in spring (13.3%) as Influenza is an ILI, so most cases occur in winter followed by autumn. Regarding the results of direct immunofluorescence test (Figure 1), it detected 23 out of 75 cases and cell culture detected 22 out of 75.

Table 4

i. Sensitivity=90.9%

ii. Specificity=94.3%

iii. Accuracy=93.3%

iv. Positive predictive value=86.9%

v. Negative predictive value=96.1%

Table 3 Frequency table show distribution of cases in relation to seasons

Season (n=75)	No	%
Autumn (September, October, November)	21	28
Winter (December, January, February)	44	58.7
Spring (March, April, May)	10	13.3
Summer (June, July, August)	0	0

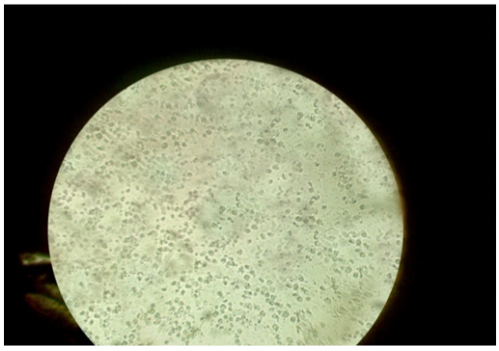

Figure 1 CPE of influenza virus on MDCK cells.

From (Table 4) we can notice that IF is sensitive (90.9%) and specific (94.3%).

Table 4 Comparison of Immuno-fluorescence staining test directly on samples and detection of CPE (cytopathogenic effect) of influenza viruses by inverted microscope

Cell Culture/IF	+ve	-ve	Total
+ve	20	3	23
-ve	2	50	52
Total	22	53	75

Age group 45-65 years was the most common age group affected by influenza A 7 (26.9%) followed by age groups <2 years & 15-30 years 3 (15.4%) for each group , then age groups 2-15 years & 30-45 years 2 (11.5%) for each group. While for influenza B , the most commonly affected age groups were <2 years & 2-15 years 2 (7.7%) for each group, followed by age group 15-30 years 1 (3.8%) with no evident influenza B positive patients in both age groups 30-45 years & 45-60 years. On comparing typing of influenza viruses by immunofluorescence directly on the samples and after tissue culture there were 3 cases of influenza type A missed before isolation. Regarding to the symptoms associated with influenza virus infection, 23 (88.5%) were associated with fever, 10 (38.5%) sore throat, 22 (84.6%) cough, 20 (76.9%) rhinorrhea, 12 (46.2%) malaise, 6 (23.1%) chills, 4 (15.4%) headache and 2 (7.7%) GIT symptoms. Rhinorrhea was significantly associated with influenza virus infection (P= 0.01).

From (Table 6) we notice that out of 12 patients with comorbidity (chronic lung disease, cardiac disease & diabetes mellitus), 9 (36.0%) were +ve for influenza virus infection (P= 0.00). The most common comorbidity with influenza virus infection was chronic lung diseases 5 (55.6%) then diabetes mellitus and cardiac diseases 2 (22.2%) for each. Regarding seasonal distribution influenza virus infection was common in winter 15 (57.7%), followed by Autumn 9 (34.6%), but this was statistically non significant. All vaccinated patients were influenza -ve and the relation between previous vaccination and influenza virus infection was statistically significant (P of fisherman exact test = 0.02). RT-PCR results obtained from nasal and throat swabs to detect influenza A virus show that 36% of nasal swabs were conventional RT-PCR positive while 32% of throat swabs were conventional RT-PCR positive (Figure 1).

NEG (NTC) - Sample cancelled due to NTC Threshold.

NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

This report generated by Rotor-Gene Q Series Software 1.7 (Build 94).

Copyright 2008 Corbett Life Science, a QIAGEN Company. All rights reserved. ISO 9001: 2000 (Reg. No: QEC21313) (Figure 2).

Real time PCR detected more positive samples when compared to conventional RT-PCR. There is direct correlation between throat and nasal swabs real time RT-PCR and this is statistically significant. The mean, median, standard deviation and range of viral load by nasal swabs are higher than those of throat swabs denoting that nasal swabs are more useful diagnostic tools in diagnosis of influenza than throat swabs (Table 5,7). Conventional reverse transcription PCR reaction on RNA extracts (Figure 3).

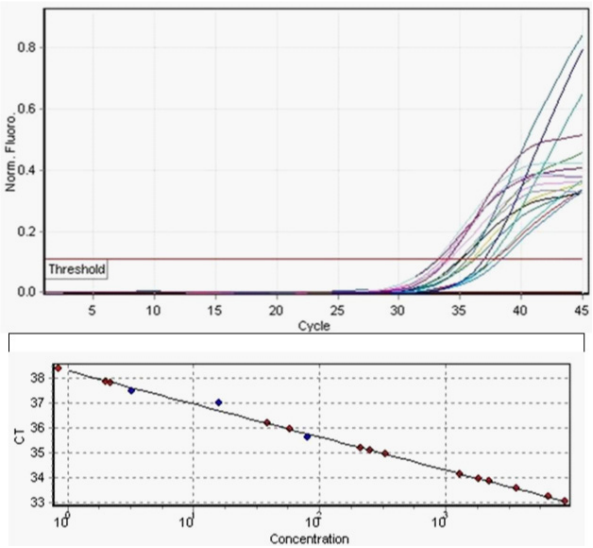

Figure 2 Standard curve RT- PCR.

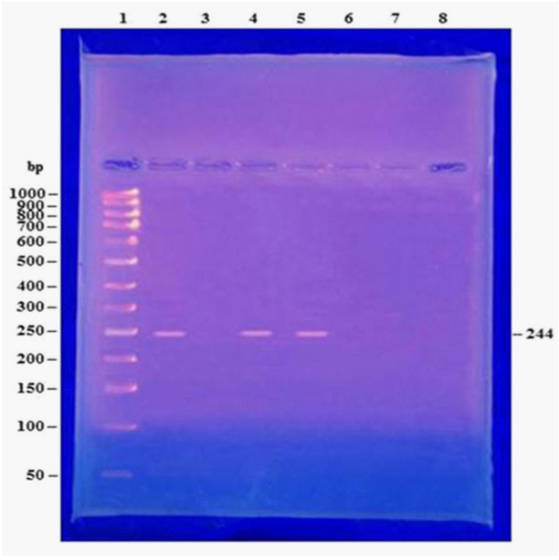

Figure 3 244 bp band size of M gene of influenza A virus.

Table 5 Relationship between throat swab conventional RT-PCR and nasal swab conventional RT-PCR

Throat Swab Nasal Swab	-ve		+ve		P of Fisher Exact Test	Kappa ±SE
	No	%	No	%		
+ve	2	11.8	7	87.5	0.000*HS	0.73±0.19
-ve	15	88.2	1	12.5		

Table 6 Nasal swab conventional RT-PCR- throat swab conventional RT-PCR kappa agreement

Nasal Swab PCR Throat Swab PCR	-ve	+ve	kappa±SE	P Value
-ve	17	2	0.74±0.1	0.00*
+ve	1	7		

Table 7a Cnmomparison of positive samples obtained by q PCR and conventional RT-PCR (total No=50) Q PCR

Q PCR Conventional RT-PCR	+ve	-ve	Sensitivity	Specificity	Predictive Value +ve	Predictive Value -ve
+ve	16	0	94.1%	100.0%	100.0%	97.1%
-ve	1	33				

Table 7b Comparison of positive samples obtained by Q PCR and conventional RT-PCR (total no=50) Conventional RT-PCR

Conventional RT-PCR Q PCR	+ve	-ve	Sensitivity	Specificity	Predictive Value +ve	Predictive Value -ve
+ve	16	1	100.0%	97.1%	94.1%	100.0%
-ve	0	33				

Discussion

Hence this study was designed to diagnose influenza virus infection as a cause of influenza disease and to compare Immunofluorescence as a rapid technique for diagnosis of influenza virus infection versus the cell culture as a gold standard for its diagnosis.[13] in our study, the sociodemographic data of influenza cases revealed that there was male predominance (61.3%).[14-16] this variation in the distribution of gender among patients with Influenza disease was explained by Muscatello et al.[17] who declared that sex was not associated with influenza disease. Concerning the isolated influenza viruses revealed by cell culture, total viruses were 22 (29.3%). This was in agreement with Clavijo et al.[12] A higher isolation rate was reported by Lopez Roa et al.[18] (40%) this may be due to the use of both MDCK and A549 cell lines.

Regarding to the isolated influenza virus, we found ,by using IF technique, that Influenza A virus was detected in 17 (77.3%) cases out of the total isolated viruses and this was more common than influenza

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B virus which were only 5 (22.7%).[18] However in Quach et al.[19] study, they isolated 48 out of 300 samples for type B and only 5 samples for type A by immunofluorescence test. This may be explained as influenza varies from season to season as well as from community to another due to differences in characteristics in the currently circulating strains.

On comparing the results of cell culture which was considered the gold standard, as reported by WHO (2007),[20] and immuono fluorescence directly on samples, we found that IF detected 23 influenza viruses (both A and B) giving the test 90.9% sensitivity, 94.3% specificity and 93.3% accuracy[21,22] who reported from different studies that the sensitivity is 70-90% and the specificity is more than 90% and concluded that IF is an important and accurate method in the detection of influenza virus. In contrast with Waner et al.[11] study, they revealed that IF was 100% sensitive and 98.1% specific also Habib-Bein et al.[23] reported that IF was 98.7% sensitive and 100% specific comparing to the results of culture. This contrast in sensitivity and specificity can be explained by difference in types of samples taken in each study. Furthermore, in our study 4 samples positive by IF but couldn't be isolated by viral isolation (false-positive); the positive predictive value was 86.9% as Waner et al.[11] and 3 samples were negative by IF and positive by cell culture (false-negative); negative predictive value 96.1% however Waner et al.[11] found no false negative IF tests.

The false negative results may be due to borderline insufficient numbers of respiratory columnar epithelial cells in the specimen. Also IF reading; is labor-intensive; and is, ultimately, subjective. For all these reasons, the results of IF are highly variable among laboratories.[23] On comparing the results of immunofluorescence before and after cell culture, we found it statistically non significant so we can use immunofluorescence directly on samples for rapid detection of influenza virus infection as it is sensitive, specific, less time consuming and more economic. As regard age distribution of positive influenza virus cases by virus isolation technique then IF staining, we found that the most affected age group was (<15) with 42.3% and the least affected age group is (45-65) with 26.9%. This finding was in agreement Zambon et al.[24] reported the same distribution but with higher percentages of 81and 29 respectively. These higher percentages may be due to using multiplex reverse transcription PCR for influenza A and B viruses.

Also Karageorgopoulos et al.[25] found that children in school age (5-15 years) were more susceptible to influenza virus infection. This may be attributed to that school-age children have the highest contact rates and they have less immunity to influenza than adults and tend to excrete large amounts of virus. Another explanation of this age distribution is the higher vaccination coverage among older adults or the duration of viral shedding in young children which is usually longer than in adults and characterized by higher virus concentration.[26] Also sample collection may be more difficult in adults than in children since adults may resist more strongly the taking of swabs or blow their noses more often and thereby reduce the quantity of virus in the nasopharyngeal swab.[27] Age group (<2) & (2-15) was the most affected group by influenza B, representing 7.7% for each group, no evident influenza B positive patients in both age groups (30-45) & (45-60). This was in accordance with Renois et al.[15] who reported that influenza B was mostly detected (<15). As regard influenza A age group (45-65) was the most common affected group (26.9%) our findings were not in agreement with Renois et al.[15] who reported that influenza A more common in (<15 years) & (15-30 years) age groups. This may be explained by increased high risk factors (chronic lung diseases, diabetes mellitus & cardiac diseases) in our study among (45-65 years) age group. Concerning the clinical presentations of influenza virus infections fever (88.5%) and cough (84.6%) were the most common presenting symptoms followed by Rhinorrhra (76.9%) and whereas GIT symptoms appeared as uncommon clinical finding (7.7%). This was in agreement with Puzelli et al.[26] & Ohmit and Monto[28] who reported that fever and cough are the best predictors for influenza virus infections. Fifteen (57.7%) of positive cases for influenza virus infection occurred in winter with peak in January 2011.[16,26] This may be due to rain fall, coldness, and contacts among children in schools.

No influenza viruses isolated from the vaccinated individuals,[29] But in Puzelli et al.[26] study a different results were found as 23 out of 118 (19.5%) were infected by influenza viruses and they attributed this finding to viral drift. From this study we conclude that the results of immuno fluorescence before and after cell culture revealed no statistically significant difference, so we recommend the use of immunofluorescence directly on nasopharyngeal samples for rapid detection of influenza virus. From our study, all vaccinated cases were negative for influenza virus, so it's recommended for high risk. The real time RT-PCR results obtained from nasal and throat swabs to detect influenza-A virus shows that 36% of nasal swabs were RT-PCR positive while 32% of throat swabs were RT-PCR positive as shown in (Table 8). This table shows real time RT-PCR results obtained from nasal swabs and throat swabs to detect influenza-A virus showing 36% of nasal swabs were RT-PCR positive while 32% of throat swabs were RT-PCR positive. The mean, median, standard deviation and range of viral load by nasal swabs are higher than those of throat swabs denoting that nasal swabs are more useful diagnostic tools in diagnosis of influenza than throat swabs.

Table 8 Comparison of viral load obtained by Q PCR for nasal and throat swabs

Viral Load	Nasal Swabs real time PCR	Throat Swabs real time PCR	Mann-Whitney U test	P Value
Mean	726.1	415.4	37.5	0.82 (non-sig.)
Median	2.02	0		
Standard deviation	1766.2	1254.2		
Range	0-8000.8	0-6000.5		

Acknowledgment

None.

Conflict of interest

None.

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