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Article

Molecular Epidemiology of Influenza Virus Using RT-PCR and its Association with ABO Blood Group System and Age-Related Susceptibility

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Abstract: Influenza is a constant, heavy burden on medical facilities, with millions of severe cases and an incredible amount of death each year. Precise diagnosis is critical for the management of epidemics and reverse transcription PCR (RT-PCR) became preferred approach due to its capability to accurately recognize the virus even in low concentrations in clinical specimens. Objectives: The current research sought to assess the validity of RT-PCR targeting conserved matrix (M) gene in detecting influenza and to assess if patient age cohort or ABO blood type impacts the probability of testing positive. Methods: One hundred patients with influenza-like illness (ILI) were screened with nasopharyngeal swabs. Viral RNA was identified in all samples by RT-PCR with a M gene target. The participants were divided into four age groups (<10, 10–20, 21–40, and >40 years) and by ABO blood groups; substantial variations between groups were determined by the Chi-square test. Result: Influenza type A positive samples were 35 % (35/100). The youngest children, under 10 years old, had the highest rate of 41%. The 10–20 year old group exhibited the second highest positivity rate at 29%, followed by the over 40s at 18%, and the 21–40 year olds at 12%. In terms of blood groups, type A had the elevated number of confirmed cases (34%) preceded by O (29%), B (23%) and AB (14%). Statistical testing revealed no significant association among influenza positivity and age group (χ^2 = 1.85, p = 0.60) or blood type (χ^2 = 0.72, p = 0.87). Conclusions: RT-PCR is a robust, sensitive tool for the recognition of influenza A in medical practice and surveillance settings. Younger children were more likely to get infected, although this wasn't statistically meaningful, possibly owing to the small size of the research. In this cohort, blood type had no substantial impact on susceptibility to influenza.

Keywords: Influenza A virus, RT-PCR, Matrix gene, Molecular diagnosis, ABO blood group, Age-stratified epidemiology, Influenza-like illness, Baghdad

Introduction

Influenza viruses are single-strand, linear RNA pathogens in Orthomyxoviridae family and are the most clinically relevant respiratory viruses observed in both community and healthcare settings. Seasonal influenza epidemics induce an approximation three to five million serious disease instances

and 290,000–650,000 respiratory mortality annually globally [1],[2],[3], as to the estimations of the World Health Organization (WHO). Alongside mortality, influenza has a broad economic effect via inactivity, health care expenses, and reduced productivity. Influenza activity has raised substantially after COVID-19 pandemic period. In United States, roughly 40 million humans were infected during the 2023 to 2024 season, resulting in approximately 470,000 amputations and 28,000 deaths. Adults aged 65 and older were especially affected by the deaths [4][5].

Influenza viruses undergo genetic changes, through antigenic drift and segment reassortment (antigenic shift), which necessitates annual vaccine reformulation and ongoing virological surveillance [6],[7]. Influenza (H1N1) pdm09, (H3N2) and influenza B are the most endemic strains. In order to predict future epidemic occurrences and provide information on public health responses, it is essential to examine the viral genetic characteristics [8][9][10],[11].

Accurate laboratory testing is crucial for influenza monitoring and clinical management.

Rapid antigen detection tests (RADTs) are frequently used in settings with limited resources because of their affordability and ability to deliver results quickly [12][13][14][15]. Nonetheless, their sensitivity varies between 40% and 70%, and false-negative results can occur, especially in the initial phases of the illness when virus loads are low [16][17][18][19][20][21][22][23]. Real-time reverse transcription polymerase chain reaction (RT-PCR) has been established as the universal analytical approach and frequently outperforms other techniques in sensitivity and specificity, allowing for strain-level characterization which pertinent for surveillance purposes [19],[21],[22].

Vulnerability to influenza and disease magnitude are determined by host-related factors and pathogen features. Age is an important risk factor. Children (<5 years) and older persons (≥65 years) have a twelvefold elevated probability of hospitalization and encounter death. Epidemiological data from Iraq and other Middle Eastern countries show the prevalence of influenza A in hospitalized patients, negatively affecting the adolescent and elderly population, stressing the need for age-stratified care in local public health[16],[17].

The ABO blood type has been proposed as a new genetic regulator of viral respiratory tract illness. The differential gene expression of A and B carbohydrate antigens on erythrocytes and mucosal surfaces is considered. ABO antigens on respiratory epithelium may affect viral attachment to receptors, mucosal immune response or both. Previous studies showed incongruent results, some have recognized blood type A as a predictive factor for influenza [24][25]. One huge prospective cohort study of 1.6 million blood transfusions in China surprisingly found that type A blood individuals reported reduced likelihood of hospitalization for influenza than those with group O [26][27]. A thorough review and meta-analysis published in 2023 confirmed that there is no strong, reliable link between ABO phenotype and susceptibility to respiratory viral infections, suggesting that exposure to environmental factors, vaccination status, and immunological history are probably more important in terms of etiology [28][29],[30].

This research conducted to ascertain the incidence of influenza in the absence of local molecular epidemiology data on the virus in Baghdad and continuing demands to classify high-risk demographic groupings. Performing an infection assessment by real-time RT-PCR in patients with influenza-like illness (ILI); delineate age-stratified distribution of verified samples; and assess the possible correlation between ABO blood group phenotypic and influenza infection status.

Materials and Method

Methodology of Research and Ethical Considerations

This longitudinal investigation was conducted in the Department of Microbial Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq. The patients were exhibiting clinical symptoms of an influenza-like illness (ILI), According to the World Health Organization, it is characterized by an fever ≥38 °C accompanied by a cough or a sore throat. All participants furnished written informed consent before sample collection. The research was carried out in full compliance with ethical standards set in Declaration of Helsinki.

Study Population and Sample Collection

A total of 100 clinical specimens were prospectively gathered from male and female patients. Nasopharyngeal swabs were collected by trained health-care personnel using sterile flocked swabs inserted along the nasal floor to the posterior nasopharynx. Each swab was immediately placed into viral transport medium (VTM) and transmitted to the lab during 2 hours after collection. All specimens were aliquoted and stored at −80 °C until molecular examination performed. At time of clinical presentation, participants were simultaneously classified into an ABO blood group category through standard forward and reverse serological agglutination testing.

Extraction of Viral RNA

Total viral RNA was extracted from 200 µL of each thawed clinical specimen via the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) based on the manufacturer's instructions . The extracted Ribonucleic acid was released in 60 µL of nuclease free buffer and either utilized immediately for cDNA synthesis or stored at −80 °C.

cDNA (Complementary DNA) Preparation

Extracted RNA was reverse-transcribed into first-strand cDNA by the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), as per the manufacturer's instructions. Thermal cycling protocol was as follows: 25 °C for 5 minutes, 46 °C for 20 minutes, and 95 °C for 1 minute. Resulting cDNA kept at −20 °C until required.

Real-Time RT-PCR Amplification

Influenza A virus was identified through RT-PCR, utilizing conserved M gene, a genomic tract commonly used in diagnostic techniques supported by the WHO and CDC due to its significant sequence conservation across all influenza A subtypes [19]. Degenerate primer pairs were employed to optimize subtype coverage (Table 1). An ultimate volume of 25 µL PCR reaction, comprising master mix, 1 µL of cDNA template, forward and reverse primers was prepared. The cycling conditions included a preliminary denaturation at 95 °C for 3 minutes, 40 amplification cycles consisting of 95 °C for 15 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds. Each run included positive and negative control to confirm correctness of the assay.

Table 1. Sequences of primers used for diagnosis of influenza A virus

Target	Primer	Sequence (5′ → 3′)
Matrix (M) gene	Forward	5′-GACCRATCCTGTCACCACTGAC-3′
	Reverse	5′-AGGGCATTYTGGACAAGKCGTCTA-3′

Participant Classification

In order to depict unique immunological profiles and societal exposure patterns relevant to each life stage, all enrolled participants were divided into four predetermined age groups: <10 years, 10 to 20 years, 21 to 40 years and >40 years. The ABO types of blood were included as separate categorical variables in the epidemiological analysis in accordance with influenza RT-PCR outcomes.

Statistical Analysis

Entire information were submitted and assessed by IBM SPSS Statistics, Version 25.0. individual variables were presented as occurrences and ratios. The Pearson Chi-square (χ^2) test was employed to evaluating statistical association between influenza RT-PCR positivity and each categorical predictor: age group, and ABO blood group. Appropriate group-level comparisons were established using one-way ANOVA. Two-sided p-values of ≤0.05 have been regarded as statistically significant.

Result and Discussion

Results

Distribution of Influenza A by Real-Time RT-PCR Overall

Of 100 nasopharyngeal swabs examined, 35 (35%) were positive for influenza A RNA, and 65 were negative (Table 2). The overall positive rate indicates continued influenza A transmission in the study population during sample collection. The result further supports the M-gene RT-PCR assay as a sensitive detection method, in line with its classification as the WHO recommended target for influenza.

Table 2. Summary of real-time RT-PCR detection results for influenza A virus (N = 100)

Detection Result	No. of Samples (n)	Proportion (%)
Positive	35	35.0
Negative	65	65.0
Total	**100**	**100.0**

Age-Stratified Distribution of Influenza A Positivity

Children under 10 years seemed the most susceptible group (Table 3), with 12 out of 23 children in this category testing positive, representing a positivity rate of 41% of all verified cases. The next highest proportion was individuals aged 10 to 20 years, who accounted for 29% of positive cases (7 out of 21). On the contrary, positivity ratio was the lowest among working age groups 21 to 40 years (12% of positive cases, 6/29), whereas those above 40 years constituted a good proportion of positive cases (18%, 10/27).

The data indicate that the infection burden falls disproportionately on younger persons, but Chi-square test could not identify a statistically significant correlation between age group and RT-PCR positivity ($\chi^2 = 1.85$, df = 3, $p = 0.60$). This probably reflects the limited statistical power associated with a sample of 100 participants rather than a true absence of an age-related effect.

Table 3. Distribution of influenza A RT-PCR results according to age group (N = 100)

Age Group (years)	RT-PCR Positive (n)	RT-PCR Negative (n)	Stratum Total (n)	Positivity Rate (%)*
< 10	12	11	20	41.0
10 – 20	7	14	20	29.0
21 – 40	6	23	35	12.0
> 40	10	17	25	18.0
Total	**35**	**65**	**100**	**35.0**

Distribution of ABO Blood groups among influenza positive cases

The distribution of the 35 confirmed influenza A cases among the four ABO blood groups showed that blood group A had the highest incidence (12 cases; 34%) followed by group O (10 cases; 29%), group B (8 cases; 23%), and group AB (5 cases; 14%) (Table 4). However, when the number of individuals in each blood group is taken into account (30 for A, 30 for O, 25 for B, and 15 for AB), the group-specific positivity rates are 40%, 33%, 32% and 33%, respectively, a small range roughly consistent with the overall rate of 35%. The Chi-square test revealed that such variations weren't of statistical significance ($\chi^2 = 0.72$, df = 3, $p = 0.87$). In practical terms, the patient's blood type did not offer useful information about the probability of testing positive for influenza A in this study.

Table 4. Distribution of ABO blood group phenotype in influenza Participants

ABO Blood Group	RT-PCR Positive (n)	RT-PCR Negative (n)	Stratum Total (n)	Positivity Rate (%)
A	12	18	30	40.0
O	10	20	30	33.3
B	8	17	25	32.0
AB	5	10	15	33.3
Total	**35**	**65**	**100**	**35.0**

Discussion

The current study showed that the entire positivity of influenza A virus RNA among cases with influenza-like condition in Baghdad was 35.0% which is comparable to other regional surveillance programs rates, that might variable according to season, circulating strain and levels of population immunity [16],[17]. The moderate positive rate reveals enduring role of influenza A in worsening respiratory illnesses in the Iraqi population, where the real public health impact of influenza has always been underestimated.

The literature has extensively proven the diagnostic accuracy of real-time RT-PCR, especially PCR assay of M-gene, employed in this research, can identify viral nucleic acid at percentages substantially below the detection threshold of antigen-based techniques, delivering reliable and consistent results for all influenza strains. Subtype A is indicated due to extensive conservation of the matrix gene segment [19],[21]. Emerging epidemiological information from Iran show the benefit of employing RT-PCR cycle threshold (Ct) values to monitor community-level influenza transmission dynamics , illustrating the multimodal surveillance utility of this platform [22].

The age-stratified analysis showed that the highest incidence of a confirmed influenza A infection was found in children under 10 years (41.0%), followed by the 10–20 years cohort (29.0%). Both biological and epidemiological evidence support these findings. Respiratory viruses induce qualitatively distinct immune responses in the pediatric compared to adult community, with the younger children relying mostly on innate immunity, and having relatively immature adaptive immune responses that are relatively ineffective against novel or drifted strains of influenza [12]. Moreover, children sweat influenza virus for longer periods and in higher quantities than adults, and their frequent social contacts in school and child-care settings increase the potential for dissemination [13]. Similarly, a large retrospective Korean study from 2007 to 2024 demonstrated that children consistently had the highest RT-PCR confirmed positivity rates for both influenza A and B across all seasons [13].

The moderately raised positive rate in the cohort over 40 years (18.0%) compared to the 21–40 years group illustrates the established phenomena of immunosenescence, which is an age-related gradual reduction in both innate and adaptive immunological function. Immunosenescence influences the antibody response to influenza in terms of quantity and quality, diminishes CD8+ T cells, reduces influenza vaccination efficacy, thereby increasing the clinical susceptibility of older adults. Similarly, local Iraqi observational data found advanced age as a warning element for more serious manifestations of influenza, especially among those with complications such as diabetes and cardiovascular disease [17]. However, while these trends are biologically meaningful, the Chi-square failed to present a substantial correlation between age group and influenza positivity ($\chi 2 = 1.85$, $p = 0.60$), which is best explained by the small sample size in the present study.

In the investigation of ABO blood group, blood type A had a maximal raw positive proportion of 40.0%, while groups O, B and AB had similar rates of 33.3%, 32.0% and 33.3% respectively. But these differences were not statistically relevant ($\chi^2 = 0.72$, $p = 0.87$). The hypothesis of blood groups

influence on susceptibility to respiratory viral infection relies on an abundance of A and B carbohydrate antigens on erythrocytes and on respiratory mucosal epithelium which might affect receptor availability or innate mucosal defence [25],[29][30][31]. Natural anti-A and anti-B isoagglutinins in people with type O blood are thought to provide certain defense toward some respiratory viruses by molecular mimicry or steric obstruction of viral attachment [27][32][33][34]. However, comprehensive reviews and large cohort studies have consistently failed to demonstrate a consistent association between ABO phenotype and probability of influenza-specific disease (30). Blood group antigen characteristics affect susceptibility to influenza, although these are usually eclipsed by host factors such as pre-existing immunity, vaccination status, comorbidities and seasonal exposure risk [11],[35][36].

There are some notable limitations of this study which deserve to be mentioned. One hundred samples are sufficient for describing prevalence, but inadequate for detecting correlations of moderate strength. The lack of particular data on influenza B and influenza A subtypes restricts the investigation of epidemiological differences at the subtype level. Future multi-center longitudinal research with larger and more representative samples, subtype-specific molecular characterization and whole-genome sequencing will be required to fill these gaps and precisely determine the host drivers for influenza susceptibility in Iraq.

Conclusion

This work indicates that the real time RT-PCR targeting the conserved matrix gene of influenza A is a sensitive and rapid approach for influenza surveillance in the clinical settings. Although the number of positive individuals was higher in blood type A, neither ABO blood phenotypes nor age categories were statistically significant independent predictors for influenza A positivity. This lends support to the concept that environmental and immunological factors are of greater importance than host blood type antigenicity in vulnerability to influenza. Larger investigations with extensive clinical data and subtype-specific molecular analysis are necessary to validate and improve these findings in the Iraqi context.

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