Influenza B viral load analysis in patients with acute respiratory infection from a tertiary hospital in Brazil

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Abstract
Currently, 2 genotypes of Influenza B viruses (IFB) are cocirculating in humans: Victoria (VIC) and Yamagata (YAM). Infection and viral load (VL) were analyzed in 105 genotyped IFB (59 VIC and 46 YAM) out of 3452 respiratory samples from immunodepressed (ID), immunocompetent (IC) including outpatients (OP) and hospitalized patients (HP) attended during 2001-2013 at São Paulo Hospital. VL (Log_{10} RNA copies/mL) calculation was possible in 78 samples (47 VIC, 31 YAM). The age group of 12 to 18 years presented the highest detection (14.13%). Rates of infection among groups were of 3.67% (IC), 1.68% (ID), 3.50% (OP), 0.6% (HP), and VLs varied from 2.8 to 10.13 with no difference regarding age, immune status, and disease severity. From 10 OP vaccinated against influenza, 8 (7 children, 1 ID) received a matching strain shot (VIC), and 2 a monovalent influenza A H1N1pdm09. Those patients presented a VL of 6.31 ± 1.62 (mean ± SD). IFB infection rates follow an age pattern, but VL seems not to be related to frequency or clinical outcome. IFB patients with previous immunization could point to some protection for VIC infections since there was no HP. Other immunological aspects, such as lineage infection immune priming, previous infections, and vaccinations, should be further investigated.

KEYWORDS
Influenza B virus, Victoria, viral Load, Yamagata

INTRODUCTION

Influenza continues to be an important cause of morbidity and mortality worldwide, affecting large segments of the human population each year. The illness is determined by an acute respiratory tract infection (ARI), tending to spread quickly in seasonal epidemics.²

Influenza can cause mild diseases or progress to a fatal outcome, particularly for high-risk groups, including children, pregnant women, elderly, immunocompromised patients, and those with chronic underlying diseases.¹,²

Influenza B virus (IFB), classified into the Orthomyxoviridae family and Beta-influenzavirus genus, began to diverge in the 1980s and are characterized today into two genetically and antigenically distinct lineages, Victoria (VIC) and Yamagata (YAM), due to a divergence of 27 amino acids in the HA1 domain of the hemagglutinin (HA) gene.³

Since the prediction of predominant IFB lineage has been uncertain, there is a need for a quadrivalent vaccine, as it would allow vaccination campaigns to be more effective in the protection of target populations.⁴

Treatment and prevention of IFB infections worldwide are based on the use of neuraminidase inhibitors (NAIs), composing the major class of antivirals recommended for this matter. Currently, there are three NAIs licensed for use in North America and Europe. Oseltamivir was the first of this class to be released, followed by zanamivir and finally peramivir. In Brazil, the Ministry of Health
The use of oseltamivir and zanamivir for treatment of influenza infections.\textsuperscript{5–8}

Osel tamivir previously showed a lower clinical efficacy against IFB and, particularly, lower effectiveness in young children. There are limitations for zanamivir prescription and a lack of other antiviral drug classes for influenza treatment in the market.

Furthermore, a recent study showed that IFB susceptibility for baloxavir (inhibitor of the viral RNA polymerase) was approximately 3-fold lower than the observed for influenza A viruses. In this regard, it is important to understand the pathophysiology of IFB viruses and disease progression to access the best management for each patient. For this purpose, understanding the viral load (VL) dynamics may consist of an essential tool to deal with episodes of influenza viral infection.\textsuperscript{9–11}

This study aimed to evaluate the rate of infection and VL quantification in positive IFB genotyped samples in different patient groups attended at a tertiary hospital in Brazil.

2 | MATERIALS AND METHODS

2.1 Sample collection

Respiratory samples from patients presenting ARI comprised of nasopharyngeal aspirates for children under 2 years of age, nasal wash (patients attended before 2009), and nasal- and oropharyngeal swabs, for patients from 2009 to 2013, according to the instructions of the Brazilian Ministry of Health protocol for management of influenza A (H1N1) 2009 pandemics. The samples were collected from patients attended at primary care health service or specific units of the São Paulo Hospital, a tertiary hospital in the city of São Paulo, Brazil, from 2001 to 2013. Informed consent was obtained from all patients before sample collection.

Samples were collected and stored in a −80°C freezer, according to physician demand and not on a regular basis. For this matter, the collection of samples from different patient groups was not evenly distributed throughout the study period.

A total of 3452 samples were analyzed and distributed into two different groups, regarding to patient conditions: (a) immunodefpressed (ID), including hematopoietic stem cell or kidney transplantation and HIV positives; and (b) immunocompetent (IC), composed of outpatients (OP) (children, adults and healthcare workers from the general community), and hospitalized patients (HP), including children and adults with severe acute respiratory infection, suspected of influenza A H1N1pdm09 infection. IFB positive samples were also distributed into different age groups according to the World Health Organization recommendations, with modifications, as follows: <2; 2 to 4; 5 to 11; 12 to 18; 19 to 58, and ≥59 years old.\textsuperscript{12} This study was conducted in compliance with institutional guidelines and approved by the Ethics Committee of São Paulo Federal University (CEP/UNIFESP n:0904/2018).

2.2 Laboratory methods

RNA extraction of samples was carried out using the QiAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s recommendations.

2.3 IFB detection and genotyping

IFB one-step real-time RT-PCR detection and further lineage differentiation were conducted as described elsewhere.\textsuperscript{13}

2.4 Viral load calculation

To calculate the VL of positive samples, a quantitative one-step real-time RT-PCR essay aimed at the nonstructural 1 (NS1) gene was performed, with human RNaseP target as internal control of sample quality and normalizing gene of threshold cycle (Ct) values. VL was calculated in Log10 RNA copies/mL. Ct values were normalized according to the following equation: \[ Ct = \left(\frac{\text{sample Ct} \times \text{RNaseP sample Ct}}{\text{mean RNaseP Ct}}\right) \].\textsuperscript{14}

2.5 Data analysis

Statistical analysis consisted of a chi-squared test for comparing categorical values, with \( p < .05 \) being considered statistically significant. Results are presented as odds ratios with the respective 95% confidence intervals (CI). All statistical analyses were performed using GraphPad Prism v.6.01.

3 RESULTS

A total of 105 IFB positive samples (59 VIC, 46 YAM) were analyzed out of 3452 respiratory samples collected from ID, IC, OP, and HP patients.

Positive patients aged from 0.25 to 72 years old (median ± SD: 16 ± 16.8). The age group of 12 to 18 years old presented the highest detection (\( p < .0001 \)) among the different age groups analyzed (Table 1).

VL calculation was possible in 78 (71.6%) samples (47 VIC and 31 YAM), due to insufficient data regarding RNaseP values. In addition, no significant difference was observed in the mean RNaseP Ct values between the types of samples analyzed (i.e., nasopharyngeal aspirate, nasal wash, nasal- and oropharyngeal swab), age group and date of collection (\( p = .45 \), the Kruskal-Wallis test).

The age groups 5 to 11 and 12 to 18 years presented the highest IFB VIC rate of infection, with 72% and 85% positivity, respectively. Conversely, IFB YAM infections occurred more frequently among young children (<5 years) and the elderly (≥59 years). No significant
A difference was observed in VL values for VIC and YAM lineages in the different age groups analyzed ($p = .07$) (Table 1, Figure 1).

When comparing immunocompetent children ($n = 33$, 0–12 years) and adults ($n = 57$, ≥13 years), both presented similar mean VLs ($6.53 ± 1.6$, and $6.6 ± 1.75$ Log10 RNA copies/mL, respectively), with no statistical difference between them ($p = 0.88$).

Rates of infection by patient condition and group were of 3.67% (IC), 1.68% (ID), 3.50% (OP), and 0.6% (HP). VLs varied from 2.8 to 10.13 Log10 RNA copies/mL (mean ± SD: $6.33 ± 1.75$) (Table 2, Figure 2). However, no statistically significant differences were observed considering immune status (immunosuppression), disease severity (outpatient vs inpatient), lineage type or age (children vs adults). On this regard (children vs adults), VL values within patient groups were, respectively, as follows: IC, $6.53 ± 1.57$ and $6.56 ± 1.75$; ID, $6.4 ± 1.98$; OP, $6.6 ± 1.45$ and $6.64 ± 1.45$.

No statistical differences were found related to lineage distribution among analyzed groups ($p = .69$). General mean VL of infections caused by VIC ($n = 47$) and YAM ($n = 31$) were of $6.4 ± 1.8$ and $6.3 ± 1.9$.

Within all IFB positive patients, 10 (9.17%) of them had received a previous influenza vaccination: 8 (7 children between 1 to 9 years and 1 ID) were shot with a vaccine strain that matched the circulating

### Table 1: IFB detection and viral load by age groups and lineage

| Age groups (years) | Patients | IFB n (%) | Odds ratio (95% CI) | VL IFB | YAM n (%) | VL YAM | VIC n (%) | VL VIC |
|--------------------|---------|-----------|---------------------|--------|-----------|--------|-----------|--------|
| <2                 | 578     | 4 (0.69)  | 1 (ref.)            | 6.9 ± 1.42 | 2 (50) | 6.55 ± 1.49 | 2 (50) | 6.49 ± 1.70 |
| 2-4                | 263     | 12 (4.18) | 6.59 (2.11-26.64)*  | 6.6 ± 1.51 | 9 (69) | 5.64 ± 1.65 | 3 (23) | 7.16 ± 0.7 |
| 5-11               | 295     | 18 (6.10) | 8.82 (2.96-26.3)*   | 6.22 ± 1.88 | 5 (28) | 4.55 ± 1.56 | 13 (72) | 6.6 ± 1.89 |
| 12-18              | 92      | 13 (14.13)| 20.42 (6.52-63.99)* | 6.64 ± 1.56 | 2 (15) | 6.04 ± 0b  | 11 (85) | 6.62 ± 1.66 |
| 19-58              | 1991    | 54 (2.86) | 3.92 (1.41-10.87)*  | 6.25 ± 1.84 | 24 (44) | 5.88 ± 1.98 | 30 (56) | 6.0 ± 1.68 |
| ≥59                | 233     | 4 (1.72)  | 2.48 (0.61-10.01)   | 2.64 ± 0b  | 4 (100) | 2.64 ± 0b  | -       | -       |

Note. VL, viral load (mean ± SD), expressed in Log10 RNA copies/mL.
Abbreviations: CI, confidence intervals; IFB, influenza B virus; SD, standard deviation; VIC, Victoria lineage; VL, viral load; YAM, Yamagata lineage.
*significant at $p < .05$.

### Table 2: Age, VL (mean ± SD) and IFB lineage detection by patient groups

| Groupsa | IC-subgroup | Patients | Age years ± SD | YAM n (%) | VIC n (%) | Viral Loadb mean ± SD (range) |
|---------|-------------|----------|----------------|-----------|-----------|-----------------------------|
| ID      | 15          | 29 ± 17.79 | 6 (40)        | 9 (60)    | 5.42 ± 1.95 (2.75-8.4)      |
| IC      | 90          | 25 ± 13.16 | 40 (44)       | 50 (56)   | 6.56 ± 1.75 (3.43-10.1)      |
| OP      | 73          | 21 ± 16.28 | 30 (41)       | 43 (59)   | 6.61 ± 1.49 (3.25-9.1)       |
| HP      | 16          | 21 ± 20.21 | 9 (56)        | 7 (44)    | 6.28 ± 2.09 (3.43-10.1)      |

Abbreviations: IFB, influenza B virus; SD, standard deviation; VIC, Victoria lineage; VL, viral load; YAM, Yamagata lineage.
aID, immunodepressed; IC, immunocompetent; OP, outpatients; HP, hospitalized patients.
b(Log10 RNA copies/mL), VL values correspond to both lineages combined.
lineage (VIC), and 2 had received a shot only against influenza A H1N1pdm09 (monovalent vaccine). Interestingly, all patients who had received a season matching vaccine strain (VIC) were infected. The remaining 2 (influenza A H1N1pdm09 vaccinated) were infected with the YAM lineage. The mean VL of vaccinated patients was of 6.31 ± 1.62, without hospitalization.

From the 16 HP, two patients presented severe outcomes: a 19 years old female (VIC) was admitted to the Intensive Care Unit (ICU), receiving mechanical ventilatory support, and was further discharged; and a female aging 46 years (YAM), codetected with human coronavirus NL-63, with diabetes mellitus type II, has died 9 days later after presenting diabetes decompensation, hemorrhagic pneumonia, rhabdomyolysis, respiratory and renal failure, and pericarditis. Another patient, a 24 years old female (VIC), was codetected with respiratory syncytial virus but was discharged without major complications.

4 | DISCUSSION

It was shown here that patients aged from 2 to 18 years old were the most affected by the IFB virus infection, which is in line with another Brazilian study.15

The group age of 12 to 18 years presented both the highest rate (14.13%) and odds ratio of infection (23.61, 95% CI: 7.51-74.23) in comparison to the other analyzed age groups. A similar result was found in a previous study, where patients with the same age range (12-18 years) also had the highest odds ratio among all different age groups evaluated (odds ratio 22.87, 95% CI: 2.90-180.66).13 In contrast to these results, a recent study held in 2019, with hospitalized children in Canada, showed that IFB virus infections were more frequent among those within 2 to 2.5 years of age, with a detection rate of 52%.16

An epidemiological study conducted in South Africa observed that IFB YAM infections were higher in individuals with 45 to 64 years and the elderly (≥65 years), which is similar to the findings formerly described, although a higher detection rate among children with 2 to 4 years was also shown. In addition, the authors state that IFB VIC infections were predominantly detected among younger age groups. In the above-described study, the highest rate of infection was observed among those with 5 to 18 years.17

Statistically significant differences were not observed between the mean values of VL for the different analyzed groups, possibly due to the fact that inpatients have not presented a higher replication level than outpatients. This outcome diverges from a recent study,18 where ICU patients presented the highest VL when compared with the others. In addition, any significant VL differences were found in relation to immune status.

The results presented could be explained by the limited number of IFB positive samples that were analyzed in some of the groups (ID, IC, OP, and HP), which may not represent the real proportion of circulating viruses among them. Another limitation was due to the retrospective design of the study, where patients were presented to physicians with a variable time of onset symptoms, which may have contributed to interfere with the final VL analysis. On the other hand, it is worthy of note that although different types of samples were used, with up to 12 years of storage, which could possibly result in changes in the obtained VL values, no significant differences were found between the mean values of Ct RNAseP either by sample type or date of collection. This may be due to the fact that, for the present study, viral RNA was obtained from aliquots of the original samples stored at −80°C that had not undergone any freeze/thaw cycle. Under these conditions, in addition to the thermal conservation, genomic RNA is protected by the viral nucleoprotein and envelope.

Eight previous immunized patients received a vaccine containing a VIC strain and presented a matched IFB infection. A similar finding was reported in a study among young children.19 However, considering that information regarding the time of vaccination was not available, these patients could have been recently vaccinated and, therefore, did not reached the adequate protective antibody titers, which are acquired at least 2 weeks postvaccination. Nonetheless, all vaccinated patients were not later hospitalized, which could point to some immunization protection.

IFB infection rates follow an age pattern but VL seems not to contribute to the frequency or outcome of the disease. The same was observed for Yamagata and Victoria lineages.

Previously immunized patients vaccinated with that same lineage could have some protection for VIC infection since patients were not later hospitalized.

In addition, host immune conditions and severe outcomes of disease could not be related to VL in this preliminary study. Other immunological aspects such as lineage infection immune priming, previous infections, and vaccinations should be further investigated.

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CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest.

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