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Research paper

Association between single nucleotide polymorphisms in TLR4, TLR2, TLR9, VDR, NOS2 and CCL5 genes with acute viral bronchiolitis

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ABSTRACT

Background: Acute viral bronchiolitis is the leading cause of hospitalization among infants during the first year of life. Most infants hospitalized for bronchiolitis do not present risk factors and are otherwise healthy. Our objective was to determine the genetic features associated with the risk and a severe course of bronchiolitis.

Methods: We prospectively evaluated 181 infants with severe bronchiolitis admitted at three hospitals over a 2-year period, who required oxygen therapy. The control group consisted of 536 healthy adults. Patients were evaluated for the presence of comorbidities (premature birth, chronic respiratory disease, and congenital heart disease), underwent nasopharyngeal aspirate testing for virus detection by multiplex-PCR, and SNPs identification in immune response genes. Patient outcomes were assessed.

Results: We observed association between SNP rs2107538*CCL5 and bronchiolitis caused by respiratory syncytial virus (RSV) and RSV-subtype-A, and between rs1060826*NOS2 and bronchiolitis caused by rhinovirus. SNPs rs4986790*TLR4, rs1898830*TLR2, and rs2228570*VDR were associated with progression to death. SNP rs7656411*TLR2 was associated with length of oxygen use; SNPs rs352162*TLR9, rs187084*TLR9, and rs2280788*CCL5 were associated with requirement for intensive care unit admission; while SNPs rs1927911*TLR4, rs352162*TLR9, and rs2107538*CCL5 were associated with the need for mechanical ventilation.

Conclusions: Our findings provide some evidence that SNPs in CCL5 and NOS2 are associated with presence of bronchiolitis and SNPs in TLR4, TLR2, TLR9, VDR and CCL5 are associated with severity of bronchiolitis.
1. Introduction

Acute viral bronchiolitis is the leading cause of hospitalization among infants aged < 12 months. Approximately 100,000 bronchiolitis admissions occur annually in the United States, at an estimated cost of $1.73 billion (Meissner, 2016; Ralston et al., 2014; Hall et al., 2009).

Respiratory syncytial virus (RSV) is the causative agent in approximately 70% of bronchiolitis cases, and nearly all children become infected with this virus during the first two years of life (Meissner, 2016; Ralston et al., 2014; Hall et al., 2009). RSV infection ranges from a mild upper respiratory illness to severe bronchiolitis, which may require admission to the intensive care unit (ICU), mechanical ventilation, and possibly lead to death. Treatment is supportive (Meissner, 2016; Ralston et al., 2014; National Collaborating Centre for Women's and Children's Health (UK), 2015). Globally, RSV is estimated to cause 66,000 to 199,000 deaths per year among children younger than five years of age (Meissner, 2016). The second most frequent virus in bronchiolitis is Rhinovirus, which has been implicated in approximately 20% of cases, and occurs in up to 39% of cases (Meissner, 2016; Ralston et al., 2014).

Because bronchiolitis can progress from mild to severe disease it is important to recognize risk factors predisposing to severe disease. We recently published a review of these topics, which include prematurity, passive smoking, young age, absence of breastfeeding, chronic lung disease, and congenital heart disease; some of these risk factors are controversial in the literature (Alvarez et al., 2013). Some controversy also exists regarding the influence of the type of virus and the presence of codetection in the severity of bronchiolitis (Hervás et al., 2012; Weigl et al., 2004; da Silva et al., 2013a; Brand et al., 2012a).

Most infants hospitalized with bronchiolitis present with no risk factors and are otherwise healthy. This led researchers to believe that epidemiological factors are not solely responsible for determining the prevalence and severity of bronchiolitis, and that these might be influenced by genetic variability. Indeed, one study including 12,346 pairs of twins, of whom a fraction was hospitalized for RSV bronchiolitis, found a correlation of 0.66 in homozygous twins and 0.53 in dizygotic twins, estimating a genetic contribution from 16% to 20% for RSV severity (Thomsen et al., 2008).

Bronchiolitis infection is restricted to the superficial cells of the respiratory epithelium. These epithelial cells recognize RSV through specialized pattern recognition receptors known as Toll Receptors or TLR Like Receptors (TLR). The human Toll-like family of proteins consists of at least 10 members of pattern recognition receptors present in macrophages and dendritic cells that represent a critical link between immune stimulants produced by microorganisms and the initiation of host defense (Tal et al., 2004; Lögren et al., 2010; Goutaki et al., 2014; Murawski et al., 2009; Mailaparambil et al., 2008).

Toll-like receptor (TLR)4 is principally expressed in macrophages, dendritic cells, and in the other cell types. It serves as a transmembrane signaling receptor of lipopolysaccharide (LPS) from Gram-negative bacteria. TLR4 is also involved in an acute innate immune response to RSV. Previous studies have shown evidence that TLR4 is engaged in pattern recognition of RSV F glycoprotein, that TLR4 expression is activated in RSV bronchiolitis, and that genetic variation of TLR4 represents a risk factor of RSV infection (Tal et al., 2004; Lögren et al., 2010; Goutaki et al., 2014). TLR2, TLR9 and TLR10 also carry polymorphisms that have been associated with bronchiolitis (Murawski et al., 2009; Mailaparambil et al., 2008), as well as other genes such as C-C motif chemokine ligand 5 (CCL5) also known as regulated on activation, normal T cell expressed and secreted (RANTES) (Amanatidou et al., 2008), vitamin D receptor (VDR) (Kresfelder et al., 2011; Janssen et al., 2007), inducible nitric oxide synthase (NOS2), interferon alpha 5 (IFNA5), and Jun proto-oncogene, AP-1 transcription factor subunit (JUN) (Janssen et al., 2007).

The aim of this study was to determine the genetic features associated with a severe course and risk of bronchiolitis.

2. Materials and methods

2.1. Patients and control group

We prospectively evaluated all severe acute viral bronchiolitis patients aged < 2 years admitted at three hospitals, in the region of the city of Campinas, São Paulo State, in Brazil, in a 2-year period (Jan/2013 to Dec/2014), who required oxygen therapy. This was the patient group, with 181 cases. One hundred thirty-one of these patients had no comorbidities (premature birth, chronic respiratory disease, and congenital heart disease). The diagnosis of bronchiolitis was based on clinical data, using the most widely accepted definition, which considers it to be the first episode of acute respiratory distress with wheezing, preceded by upper airway symptoms such as rhinorrhea and cough, with or without fever, in children under 2 years of age (Ralston et al., 2014). The severe bronchiolitis criterion was oxygen saturation < 92%. Patients with this condition were admitted for oxygen therapy (Ralston et al., 2014). Patients with previous wheezing were excluded. Patients were admitted in ICU when oxygen saturation remains < 92% even with the patient getting inspired oxygen fraction > 60%. Patients were submitted to mechanical ventilation if arterial partial pressure of oxygen were < 60 mm Hg or arterial partial pressure of carbon dioxide were > 50 mm Hg in arterial blood gas analysis. The oxygen therapy was suspended when oxygen saturation remains > 92% in room air. The patient was discharged 24 h after suspending the oxygen therapy.

Patients were evaluated for the presence of comorbidities (premature birth, chronic respiratory disease, and congenital heart disease) and for other epidemiological variables: birth weight, gender, cesarean delivery, gestational age, breastfeeding, maternal smoking during pregnancy, passive smoke exposure, allergies in parents and siblings, number of siblings, numbers of persons in the house, mold exposure, pets in the house, down syndrome, day care attendance and mother’s years of education. Parents or guardians answered a questionnaire about epidemiological factors. Outcome of disease was studied performing a longitudinal follow-up of these patients until the time of discharge, evaluating the length of hospital stay, length of oxygen use, need and length of ICU stay, need and length of mechanical ventilation, and progression to death.

Patients underwent nasopharyngeal aspirate for the detection of viruses, and blood collection for the identification of polymorphisms.

The control group consisted of 536 healthy controls (aged 19 to 25 years), randomly invited to participate in the study, with no personal or family history of lung or other chronic disease for two generations, and was from the same geographic region as the patients group. Participants in the control group were all interviewed, and it was ruled out that they had been hospitalized in childhood for respiratory problems. In this way, we eliminated the possibility that they have presented severe acute viral bronchiolitis. In our study, no healthy controls were included from preexisting cohort, blood bank, patient population and/or patient’s parents. The study of ancestry was not performed due to the high cost of analysis. Using a control group with healthy adults is a useful and accepted tool that has been applied in a large number of genetic association studies (Tal et al., 2004; Mailaparambil et al., 2008; Amanatidou et al., 2008; Janssen et al., 2007; Arruvito et al., 2015; Ricciardolo et al., 2004).

2.2. Virus screening

RNAprotect® Cell Reagent (Qiagen, Valencia, CA, USA) was added to nasopharyngeal aspirates in a 1:5 ratio, and stored at −80 °C. Stored material was centrifuged and the supernatant discarded. The cell pellet was then resuspended in buffer RLT Plus and DNA and RNA isolation was performed using the AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol.

cDNA was synthesized using the High Capacity cDNA Reverse
Virus (RSV, specifically) was analyzed for polymorphism frequencies in patients for each type of polymorphism. The frequency was compared between patients and controls. We used statistical analysis, for all previously mentioned comparisons, was performed by using Statistical Package for the Social Sciences 21.0 software (SPSS Inc., Chicago, IL).

Polymorphism screening

The SNPs enrolled in our data were selected based on previous studies (Tal et al., 2004; Loigren et al., 2010; Goutaki et al., 2014; Murawski et al., 2009; Mailaparambil et al., 2008; Amanatidou et al., 2008; Kresfelder et al., 2011; Janssen et al., 2007; Arruvito et al., 2014; Murawski et al., 2009; Mailaparambil et al., 2008; Amanatidou et al., 2008). All SNPs studied are bi-allelic. Genomic DNA was extracted from blood samples using the QiAamp® DNA Blood Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The OpenArray® Real-Time PCR Platform and AccuFill™ System (Thermo Fisher Scientific, São Paulo, Brazil) were used to screen the following 16 polymorphisms with TaqMan® OpenArray® Genotyping Plates, format 16 (PN: 4413546): rs4986791 (TLR4), rs4986791 (TLR4), rs1927911 (TLR4), rs1898830 (TLR2), rs7656411 (TLR2), rs352162 (TLR9), rs187084 (TLR9), rs1065341 (CCL5), rs2107538 (CCL5), rs2208788 (CCL5), rs2208789 (CCL5), rs10735810 (VDR), rs2228570 (VDR), rs1060826 (NOS2), rs10757212 (IFN5A), and rs11688 (JUN).

Statistical analysis

Epidemiological and laboratory data were described by frequency (percentage) for categorical variables, and means ± standard deviation or medians (minimum and maximum) for quantitative variable data. Disease outcomes were compared between patients, and polymorphism frequency was compared between patients and controls. We also analyzed polymorphism frequencies in patients for each type of virus (RSV, specific RSV subtype A or RSV subtype B, rhinovirus, and virus codeletion) comparing each subgroup with controls. A second analysis, for all previously mentioned comparisons, was performed excluding 50 patients with comorbidities (premature birth, chronic respiratory disease, and congenital heart disease), this group remained with 131 patients. This analysis was performed to exclude the possible influence of those comorbidities in patient’s outcomes.

Fisher’s exact test and the χ² test were applied to compare categorical data. We reported odds ratios (OR) and 95% confidence intervals; two-sided P < 0.05 was considered statistically significant. Data were analyzed using Statistical Package for the Social Sciences 21.0 software (SPSS Inc., Chicago, IL).

We evaluated minor allele frequency (MAF) and Hardy-Weinberg equilibrium (HWE) using OEGE software (Rodriguez et al., 2009). In order to evaluate genetic interaction among the polymorphisms and clinical data in our sample, we used the Multifactor Dimensionality Reduction (MDR) model, which is a nonparametric, genetic, and environmental model-free data mining for nonlinear interaction identification among genetic and environmental attributes (Gola et al., 2016). To adjust results for multiple comparisons, we performed a MDR permutation test in our sample, totaling 100,000 permutations.

Details about statistical analysis:

(a) Association study models: For single nucleotide polymorphisms (SNPs) evaluation we performed comparison considering all major possibilities of genetic association, including the allelic analysis. For example, regarding one hypothetical SNP with the allele 1 (A1) and allele 2 (A2), we performed the allelic analysis + all genotypes comparisons [(i) A1A1 versus A1A2 versus A2A2; (ii) A1A1 versus A1A2 + A2A2; (iii) A1A1 + A1A2 versus A2A2; (iv) A1A2 versus A1A1 + A2A2]. We chose to present only the associations with P value < 0.05.

(b) Models for calculation of OR in the presence of codominance: OR was calculated for the three possibilities when P value were < 0.05 for co-dominant model: (i) A1A1 versus A1A2 + A2A2; (ii) A1A1 + A1A2 versus A2A2; (iii) A1A2 versus A1A1 + A2A2.

(c) Overdominance analysis: The overdominance analysis was made considering the important contribution of heterozygous genotype in maintaining the prevalence of several diseases studied in the literature, including cancer and asthma. Although many studies have reported the importance of the heterozygous genotype prevalence of different diseases, the role of heterozygosis is not well known in many cases and needs to be better understood by genetic association studies in different populations.

(d) Bonferroni correction: Association studies, including genetic variables, environmental variables (such as viral identification) and clinical data of patients and healthy controls allow the possibility of performing multiple comparisons. Bonferroni correction is a method employed to minimize the effect of multiple comparisons and the presence of false positives. We tested 16 SNPs but two showed no amplification, so Bonferroni correction was applied and correction of the P value was carried out by multiplying by 14 (α = 0.05/14). The Bonferroni correction method for multiple testing was published in 1935. Even with wide applicability and use, some authors consider unnecessary to carry out this correction, so we decided to present both data (corrected and uncorrected).

Additional references for the statistical models applied in the study was set at online supplemental material 1.

2.5 Ethics statement

This study was approved by the National Research Ethics Commission and was conducted according to the Declaration of Helsinki principles. Legal representatives of patients, and participants of the control group, received explanations about the research and gave written informed consent.

3. Results

Table 1 shows patient demographics and clinical characteristics. Table 2 shows details of the viruses identified. One hundred and eighty-one patients were included in the study, 173 of these patients underwent nasopharyngeal aspirate. Viral identification was positive in 87.3% of cases; the most frequently detected virus was RSV in 121 samples (69.9%), followed by rhinovirus, which was present in 46 samples (26.6%). One hundred and thirty-one patients had no comorbidities, 126 of these patients underwent nasopharyngeal aspirate. Viral identification was positive in 87% of cases; the most frequently detected virus in this group was RSV in 89 samples (70.6%), followed by rhinovirus, which was present in 31 samples (24.6%).

The control group was used to achieve the SNPs genotype frequency and to associate with severe acute viral bronchiolitis group.
Table 1
Demographic and clinical characteristics in 181 infants hospitalized with bronchiolitis (173 underwent nasopharyngeal aspirate). Data of the 131 infants without comorbidities is shown in a separated column (126 underwent nasopharyngeal aspirate).

| Item | All patients (181) | Patients without comorbidities (131) |
|------|--------------------|--------------------------------------|
| Birth weight (gr) | 3032.47 ± 671.79; 3102 (565 to 4850) | 3264.10 ± 474.32; 3240 (2050 to 4850) |
| Male gender | 105 (58%) | 72 (55%) |
| Cesarean delivery | 103 (58.2%) | 72 (55.8%) |
| Gestational age (weeks) | 37.42 ± 2.39; 38 (28 to 42) | 38.43 ± 1.34; 38 (37 to 42) |
| Premature birth (< 37 weeks) | 38 (21%) | – |
| Age (days) | 140.61 ± 118.13; 104 (16 to 622) | 137.16 ± 101.18; 111 (16 to 469) |
| Infants breastfed since birth | 64 (36.6%) | 25 (19.1%) |

Family and environmental data in infants hospitalized with bronchiolitis

| Item | All patients (181) | Patients without comorbidities (131) |
|------|--------------------|--------------------------------------|
| Maternal smoking during pregnancy | 18 (10.2%) | 12 (9.4%) |
| Passive smoke exposure | 50 (28.4%) | 37 (28.9%) |
| Father with asthma | 21 (11.9%) | 15 (11.6%) |
| Siblings with asthma | 38 (21.5%) | 19 (14.7%) |
| Mother with allergic rhinitis | 45 (25.6%) | 33 (25.6%) |
| Father with allergic rhinitis | 27 (15.3%) | 20 (15.5%) |
| Siblings with allergic rhinitis | 33 (18.6%) | 23 (17.8%) |
| Mother with atopic dermatitis | 8 (4.5%) | 6 (4.7%) |
| Father with atopic dermatitis | 3 (1.7%) | 2 (1.6%) |
| Siblings with atopic dermatitis | 17 (9.6%) | 11 (8.3%) |
| Number of siblings | 1.34 ± 1.31; 1 (0 to 7) | 1.19 ± 1.15; 1 (0 to 5) |
| Numbers of persons in the house | 4.52 ± 1.43; 4 (2 to 13) | 4.36 ± 1.34; 4 (2 to 13) |
| Mold exposure | 37 (21%) | 27 (21.1%) |
| Pets in the house | 79 (44.9%) | 62 (48.4%) |
| Day care attendance | 25 (14.1%) | 17 (13.2%) |
| Mothers with ≥ 10 to 16 years of education | 28 (16.2%) | 18 (14.2%) |
| Mothers with 9 years of education | 83 (48%) | 60 (47.2%) |
| Mothers with 10 to 16 years of education | 62 (35.8%) | 49 (38.6%) |
| Mothers with ≥ 17 years of education | – | – |

Comorbidities in infants hospitalized with bronchiolitis

| Item | All patients (181) | Patients without comorbidities (131) |
|------|--------------------|--------------------------------------|
| Chronic respiratory disease | 3 (1.7%) | – |
| Congenital heart disease | 13 (7.3%) | – |
| Down syndrome | 4 (2.2%) | – |

Outcome in infants hospitalized with bronchiolitis

| Item | All patients (181) | Patients without comorbidities (131) |
|------|--------------------|--------------------------------------|
| Length of hospital stay (days) | 6.5 (1 to 64) | 6 (1 to 26) |
| Length of oxygen therapy (days) | 5 (1 to 63) | 5 (1 to 24) |
| Intensive care unit (ICU) admission | 61 (34%) | 41 (32%) |
| Length of ICU stay (days) | 9 (2 to 37) | 8 (2 to 20) |
| Need of mechanical ventilation | 38 (21%) | 25 (19.5%) |
| Length of mechanical ventilation | 8.5 (2 to 37) | 8 (2 to 18) |
| Death | 5 (2.8%) | 4 (3.1%) |

Data are expressed as mean ± standard deviation; medians (range) or frequencies (percentage).

Table 2
Virus identified in 181 infants hospitalized with bronchiolitis (173 underwent nasopharyngeal aspirate). Data of the 131 infants without comorbidities is shown in a separated column (126 underwent nasopharyngeal aspirate).

| Item | All patients (181) | Patients without comorbidities (131) |
|------|--------------------|--------------------------------------|
| Respiratory syncytial virus | 121 (69.9%) | 89 (70.6%) |
| Respiratory syncytial virus A | 91 (52.6%) | 64 (50.8%) |
| Respiratory syncytial virus B | 31 (17.9%) | 25 (19.8%) |
| Rhinovirus | 46 (26.6%) | 31 (24.6%) |
| Parainfluenza virus | 6 (3.5%) | 4 (3.2%) |
| Adenovirus | 8 (4.6%) | 5 (4%) |
| Coronavirus | 3 (1.7%) | 2 (1.6%) |
| Influenza virus | 2 (1.2%) | – |
| Bocavirus | 1 (0.6%) | – |
| Metapneumovirus | 3 (1.7%) | 2 (1.6%) |
| Enterovirus | 2 (1.2%) | 2 (1.6%) |
| Negative | 22 (12.7%) | 17 (13%) |
| Codetection | 37 (21.4%) | 26 (20.6%) |

Data are expressed as frequencies (percentage).

3.1. Association between polymorphisms and bronchiolitis presence and severity

Table 3 shows SNPs description including MAF and HWE. SNPs rs10735810 (VDR) and rs116688 (JUN) assays failed in our test. SNPs rs1065341 and rs2280788 (CCL5) are not in HWE for patients and controls subjects. Moreover, SNP rs1060826 (NOS2) is not in HWE only for controls subjects.

Fig. 1 and Table 4 show association between polymorphisms and the presence of bronchiolitis for different types of virus. The evaluation of the presence of bronchiolitis for different types of virus revealed an association between SNP rs2107538*CT (CCL5) and bronchiolitis caused by RSV (OR = 1.646; 95%CI = 1.054 to 2.57) and RSV subtype A (OR = 1.754; 95%CI = 1.06 to 2.902) specifically, and between SNP rs1060826*GG (NOS2) and bronchiolitis caused by rhinovirus (OR = 2.649; 95%CI = 1.309 to 5.362). In patients without comorbidities, we observed an association between SNP rs1060826*GG (NOS2) and bronchiolitis caused by rhinovirus (OR = 3.369; 95%CI = 1.812 to 6.274) and between SNP rs187084*TC (TLR9) (OR = 0.455; 95%CI = 0.222 to 0.935) with ICU admission, and rs352162*CC + CT (TLR9) (OR = 2.718; 95%CI = 1.103 to 6.695) and rs2107538*TT (CCL5) (OR = 4.974; 95%CI = 1.047 to 23.63) with need for mechanical ventilation.
In patients without comorbidities, SNP rs1898830 (TLR2) was associated with progression to death because the four patients who died during the study were heterozygous for this SNP (P = 0.036). Additionally, rs1927911*CC (TLR4) (OR = 3.578; 95%CI = 1.238 to 10.34) and rs2107538*TT (CCL5) (OR = 6; 95%CI = 1.221 to 29.48) were associated with need for mechanical ventilation, rs7656411*GT (TLR2) (OR = 2.5; 95%CI = 1.093 to 5.718) with length of oxygen use, and rs352162*TT (TLR9) (OR = 4.886; 95%CI = 1.689 to 14.13) and rs2280788 (CCL5) with ICU admission. The rs2280788 (CCL5) heterozygous genotype was only present in patients who required ICU admission (P = 0.036).

After Bonferroni correction, the following associations maintained P < 0.05: rs1060826*CC + TT (NOS2) and patients with Rhinovirus infection (Fig. 1, Table 4); rs352162*TT (TLR9) and ICU admission (in all patients group) (Fig. 2, Table 5).

### Table 3

| Gene | SNPs  | Ancestral allele (AA) | Rare allele (RA) | Functional consequence | Amino acid AA  | Amino acid RA  | Group | MAF    | HW     |
|------|-------|-----------------------|------------------|-----------------------|----------------|----------------|-------|--------|--------|
| TLR4 | rs4986790 | A                     | G                | Missense             | Asp            | Gly            | SAVB  | 0.09 (G) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.07 (G) | > 0.05 |
|      | rs4986791 | C                     | T                | Missense             | Thr            | Ile            | SAVB  | 0.02 (T) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.05 (T) | > 0.05 |
|      | rs1927911 | T                     | C                | Intron variant       |                |                | SAVB  | 0.34 (T) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.33 (T) | > 0.05 |
| TLR2 | rs11986830 | C                     | A                | Intron variant       |                |                | SAVB  | 0.34 (C) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.34 (C) | > 0.05 |
|      | rs7656411 | G                     | T                | Downstream variant 500B |                |                | SAVB  | 0.34 (G) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.34 (G) | > 0.05 |
| TLR9 | rs552162 | C                     | T                |                         |                |                | SAVB  | 0.47 (T) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.46 (T) | > 0.05 |
|      | rs187084 | C                     | T                | Upstream variant 2 KB  |                |                | SAVB  | 0.39 (C) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.42 (C) | > 0.05 |
| CCL5 | rs1065341 | A                     | G                | Intron variant, UTR 3 prime |                |                | SAVB  | 0.49 (G) | < 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.48 (G) | < 0.05 |
|      | rs2107538 | T                     | C                | Upstream variant 2 KB  |                |                | SAVB  | 0.28 (T) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.27 (T) | > 0.05 |
|      | rs2280788 | C                     | G                | Intron variant, Upstream variant 2 KB |                |                | SAVB  | 0.43 (G) | < 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.38 (G) | < 0.05 |
|      | rs2280789 | C                     | T                | Intron variant       |                |                | SAVB  | 0.2 (C)  | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.18 (C) | > 0.05 |
| VDR  | rs10735810 | T                    | C                | Missense             | Met            | Thr            | SAVB  | –      | –      |
|      |        |                       |                  |                       |                |                | Control | –      | –      |
|      | rs2228570 | T                     | C                | Missense             | Met            | Thr            | SAVB  | 0.33 (T) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.33 (T) | < 0.05 |
| NOS2 | rs1066826 | G                     | A                | Synonymous codon     | Thr            | Thr            | SAVB  | 0.33 (A) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.35 (A) | > 0.05 |
| IFNA5 | rs10757212 | A                    | G                | Synonymous codon     | Thr            | Thr            | SAVB  | 0.3 (A) | > 0.05 |
|      |        |                       |                  |                       |                |                | Control | 0.3 (A) | > 0.05 |
| JUN  | rs11688  | G                     | A                | Synonymous codon     | Gln            | Gln            | SAVB  | –      | –      |
|      |        |                       |                  |                       |                |                | Control | –      | –      |

AA, ancestral allele; RA, rare allele; SNP, single nucleotide polymorphism; MAF, minor allele frequency; HW, Hardy-Weinberg Equilibrium; TLR4, Toll-like receptor 4; TLR2, Toll-like receptor 2; TLR9, Toll-like receptor 9; CCL5, C-C motif chemokine ligand 5; VDR, Vitamin D Receptor; NOS2, Inducible nitric oxide synthase; IFNA5, interferon alpha 5; JUN, Jun proto-oncogene, AP-1 transcription factor subunit; SAVB, severe acute viral bronchiolitis; UTR, untranslated region; (-), assays that failed in our test.

In bold type shown the SNPs that are not in Hardy-Weinberg equilibrium.

* Data obtained from NCBI including the information about ancestral and rare alleles.

In patients without comorbidities, SNP rs1898830 (TLR2) was associated with progression to death because the four patients who died during the study were heterozygous for this SNP (P = 0.036). Additionally, rs1927911*CC (TLR4) (OR = 3.578; 95%CI = 1.238 to 10.34) and rs2107538*TT (CCL5) (OR = 6; 95%CI = 1.221 to 29.48) were associated with need for mechanical ventilation, rs7656411*GT (TLR2) (OR = 2.5; 95%CI = 1.093 to 5.718) with length of oxygen use, and rs352162*TT (TLR9) (OR = 4.886; 95%CI = 1.689 to 14.13) and rs2280788 (CCL5) with ICU admission. The rs2280788 (CCL5) heterozygous genotype was only present in patients who required ICU admission (P = 0.036).

After Bonferroni correction, the following associations maintained P < 0.05: rs1060826*CC + TT (NOS2) and patients with Rhinovirus infection (Fig. 1, Table 4); rs352162*TT (TLR9) and ICU admission (in all patients group) (Fig. 2, Table 5).

**Fig. 1.** Association between polymorphisms and presence of bronchiolitis for different types of virus, data for all patients and patients without comorbidities. RSV, respiratory syncytial virus; OR, odds ratio; CI, confidence interval. All parameters show significant differences between or among groups before Bonferroni correction (P < 0.05). The SNP rs1060826*CC + TT shows significant difference between groups after Bonferroni correction (P < 0.05) in patients with Rhinovirus infection. Fisher’s exact test and the χ² test were applied considering data distribution. The statistical model applied in genetics analysis, considering the SNP genotype used for odds ratio calculation, is presented in parentheses. The MDR analysis showed no evidence of interaction of genetic data with acute severe viral bronchiolitis for the positive association.
Table 4
Associations between polymorphisms and presence of bronchiolitis for different types of virus, data for all patients and patients without comorbidities.

### All patients

| Polymorphism       | Yes  | Control group | Total | Odds ratio | 95%CI    |
|--------------------|------|---------------|-------|------------|----------|
| RSV rs2107538 (CCL5) |      |               |       |            |          |
| CT                 | 48   | 170           | 218   | 1.646      | 1.054 to 2.57 |
| CC + TT            | 47   | 274           | 321   | 1          | –        |
| RSV subtype A rs2107538 (CCL5) |      |               |       |            |          |
| CT                 | 37   | 170           | 207   | 1.754      | 1.06 to 2.902  |
| CC + TT            | 34   | 274           | 308   | 1          | –        |
| Rhinovirus rs1060826 (NOS2) |      |               |       |            |          |
| GG                 | 23   | 185           | 208   | 2.649      | 1.309 to 5.362  |
| GA + AA            | 13   | 277           | 290   | 1          | –        |
| Rhinovirus rs1060826 (NOS2) |      |               |       |            |          |
| GG + AA            | 28   | 234           | 262   | 3.41       | 1.522 to 7.64  |
| GA                 | 8    | 228           | 236   | 1          | –        |

### Patients without comorbidities

| Polymorphism       | Rhinovirus | Total | Odds ratio | 95%CI    |
|--------------------|------------|-------|------------|----------|
|                    | Yes        | Control group |       |          |
| rs1060826 (NOS2)   |            |               |       |          |
| GG                 | 18         | 185           | 203   | 3.369     | 1.435 to 7.908  |
| GA                 | 5          | 228           | 233   | 0.244     | 0.091 to 0.659  |
| AA                 | 3          | 49            | 52    | 1.099     | 0.318 to 3.795  |

RSV, respiratory syncytial virus; CI, confidence interval; CCL5, C-C motif chemokine ligand 5; NOS2, Inducible nitric oxide synthase. All parameters show significant differences between or among groups before Bonferroni correction (P < 0.05).

* The types of virus were screened only in the patients with bronchiolitis.

* Parameter shows significant difference between groups after Bonferroni correction (P < 0.05). Fisher's exact test and the $\chi^2$ test were applied considering the data distribution. The MDR analysis showed no evidence of interaction of genetic and viral data with acute severe viral bronchiolitis for the positive association (data not showed).
3.2. Interaction analysis

The MDR analysis showed no evidence of interaction of genetic variants enrolled between severe acute viral bronchiolitis and healthy control. For patient’s outcomes, no positive interaction was observed among the clinical data.

3.3. Allelic analysis

No significant association was find between the SNPs alleles and the presence of bronchiolitis considering or not the presence of comorbidities (Tables 6, 7).

4. Discussion

To the best of our knowledge, this is the first study into the association of SNPs in genes involved in the immune response with the severity of bronchiolitis that compared the outcomes of patients that have been admitted to a hospital. Until now, studies analyzing the prevalence of bronchiolitis have compared patients with controls, while those examining the severity of bronchiolitis have compared inpatients with patients seen in the emergency room and then discharged.
Table 6

Associations between polymorphisms and presence of bronchiolitis for the allelic model.

| Gene | SNPs     | Allele | SAVB | Control | Total | P-value | OR     | 95% CI  |
|------|----------|--------|------|---------|-------|---------|--------|--------|
| TLR4 | rs4986790 | A      | 241  | 811     | 1052  | 0.491   | 0.84   | 0.511 to 1.38 |
|      |          | G      | 23   | 65      | 88    | 1       | –      | –      |
|      |          | C      | 236  | 819     | 1055  | 0.152   | 1.873  | 0.784 to 4.478 |
|      |          | T      | 6    | 39      | 45    | 1       | –      | –      |
|      |          | C      | 192  | 706     | 998   | 0.874   | 1.024  | 0.766 to 1.368 |
| TLR2 | rs1898830 | C      | 91   | 300     | 391   | 0.854   | 1.027  | 0.769 to 1.372 |
|      |          | T      | 6    | 39      | 45    | 1       | –      | –      |
|      | rs7656411 | C      | 91   | 339     | 430   | 0.357   | 0.874  | 0.657 to 1.163 |
|      |          | T      | 6    | 39      | 45    | 1       | –      | –      |
| TLR9 | rs352162  | T      | 143  | 509     | 652   | 0.767   | 0.96   | 0.731 to 1.26  |
|      |          | C      | 175  | 616     | 791   | 1       | –      | –      |
|      |          | T      | 125  | 427     | 552   | 1       | –      | –      |
|      |          | C      | 163  | 543     | 706   | 1       | –      | –      |
| CCL5 | rs1065341 | A      | 132  | 456     | 588   | 0.717   | 1.051  | 0.802 to 1.379 |
|      |          | G      | 136  | 494     | 630   | 1       | –      | –      |
|      |          | C      | 7    | 56      | 62    | 0.874   | 1.027  | 0.769 to 1.372 |
|      |          | T      | 4    | 39      | 43    | 1       | –      | –      |
|      | rs2280788 | G      | 256  | 842     | 1126  | 0.264   | 0.548  | 0.189 to 1.594 |
|      |          | C      | 131  | 616     | 747   | 1       | –      | –      |
|      |          | T      | 214  | 774     | 988   | 1       | –      | –      |
| NOS2 | rs1060826 | C      | 157  | 583     | 740   | 0.995   | 1.051  | 0.78 to 1.386  |
|      |          | T      | 130  | 427     | 560   | 1       | –      | –      |
|      | rs10757212| A      | 120  | 466     | 586   | 0.415   | 0.886  | 0.663 to 1.185 |
|      |          | G      | 186  | 705     | 891   | 1       | –      | –      |

OR, odds ratio; CI, confidence interval; TLR4, Toll-like receptor 4; TLR2, Toll-like receptor 2; TLR9, Toll-like receptor 9; CCL5, C-C motif chemokine ligand 5; VDR, Vitamin D Receptor; NOS2, Inducible nitric oxide synthase; IFNA5, interferon alpha 5; JUN, Jun proto-oncogene, AP-1 transcription factor subunit; SAVB, severe acute viral bronchiolitis; UTR, untranslated region; (-), reference. The χ² test was applied considering the data distribution.

Table 7

Associations between polymorphisms and presence of bronchiolitis for the allelic model excluding patients with comorbidities.

| Gene | SNPs     | Allele | SAVB | Control | Total | P-value | OR     | 95% CI  |
|------|----------|--------|------|---------|-------|---------|--------|--------|
| TLR4 | rs4986790 | A      | 160  | 811     | 971   | 0.323   | 0.754  | 0.431 to 1.321 |
|      |          | G      | 17   | 65      | 82    | 1       | –      | –      |
|      |          | C      | 174  | 819     | 993   | 0.162   | 2.071  | 0.731 to 5.872 |
|      |          | T      | 4    | 39      | 43    | 1       | –      | –      |
|      | rs1927911 | T      | 65   | 306     | 371   | 0.995   | 0.999  | 0.72 to 1.386  |
|      |          | C      | 131  | 616     | 747   | 1       | –      | –      |
|      | rs2280788 | G      | 256  | 842     | 1126  | 0.264   | 0.548  | 0.189 to 1.594 |
|      |          | C      | 131  | 616     | 747   | 1       | –      | –      |
|      | rs2280789 | C      | 178  | 598     | 776   | 1       | –      | –      |
|      |          | T      | 140  | 774     | 914   | 1       | –      | –      |
| VDR  | rs2228570 | T      | 88   | 315     | 403   | 0.938   | 0.989  | 0.74 to 1.319  |
|      |          | C      | 180  | 637     | 817   | 1       | –      | –      |
| NOS2 | rs1060826 | C      | 86   | 326     | 412   | 0.415   | 0.886  | 0.663 to 1.185 |
|      |          | A      | 178  | 598     | 776   | 1       | –      | –      |
| IFNA5| rs10757212| A      | 78   | 265     | 343   | 0.793   | 0.961  | 0.711 to 1.298 |
|      |          | G      | 186  | 607     | 793   | 1       | –      | –      |

OR, odds ratio; CI, confidence interval; TLR4, Toll-like receptor 4; TLR2, Toll-like receptor 2; TLR9, Toll-like receptor 9; CCL5, C-C motif chemokine ligand 5; VDR, Vitamin D Receptor; NOS2, Inducible nitric oxide synthase; IFNA5, interferon alpha 5; JUN, Jun proto-oncogene, AP-1 transcription factor subunit; SAVB, severe acute viral bronchiolitis; UTR, untranslated region; (-), reference. The χ² test was applied considering the data distribution.
4.1. Virus

RSV was present in 69.9% of patients; 52.6% of patients had RSV-A and 17.9% had RSV-B. The second most common virus was rhinovirus, which was present in 26.6% of patients. Viral codetection was present in 21.4% of the patients. Codetection has been reported to in up to 65% of patients with bronchiolitis (Rodríguez et al., 2014). Some controversy exists regarding the influence of the presence of codetection in the severity of bronchiolitis. Some studies suggest that codetection increases the severity of bronchiolitis (Rodríguez et al., 2014; da Silva et al., 2013b), while other studies have shown that patients with viral codetection do not present a more serious disease than patients infected with a single virus (Ricart et al., 2013; Brand et al., 2012b). In our study codetection was associated with patient age and frequency of nursery attendance but was not associated with bronchiolitis severity. The influence of codetection in bronchiolitis severity was not an objective of our study so we will not make an extensive discussion of this topic.

4.2. Toll like receptors

4.2.1. Toll like receptor 4 – TLR4

The interaction between TLR4 and the RSV fusion protein leads to the production of pro-inflammatory cytokines (interleukins 6, 8, 10, and 13, tumor necrosis factor, CCL5, and CX3CR1) and surfactant proteins. Some of these factors have direct antiviral properties, while others stimulate the activation of natural killer cells, granulocytes, monocytes, and macrophages, thus initiating the adaptive immune response (Farrag and Almajhdi, 2016; Arruvito et al., 2015; Lambert et al., 2014; Choi et al., 2013). A TLR deficiency may lead to the absence of Th1 polarizing signals, and change T cell responses from protective Th1 and cytotoxic T cell immunity toward dysregulated Th2 and Th17 polarization, causing bronchiolitis in susceptible infants (Farrag and Almajhdi, 2016; Arruvito et al., 2015). A recent study demonstrated that the RSV fusion protein was capable of inducing the formation of neutrophil extracellular traps (NETs), which immobilize and kill pathogens, through TLR4 activation. The excessive production of NETs contributes to the pathology of respiratory viral infections (Punchal et al., 2015).

Previous studies found that severe RSV bronchiolitis is associated with SNPs in TLR4 (rs4986790 and rs4986791) (Tal et al., 2004; Puthothu et al., 2006). Moreover, peripheral blood mononuclear cells from children expressing exonic TLR4 variants were shown to have blunted responses to RSV (Ricart et al., 2013). However, other studies found no association between these SNPs and bronchiolitis (Löfgren et al., 2010; Goutaki et al., 2014). The present study found that death from bronchiolitis is associated with SNP rs4986790 (TLR4), but no association was detected between bronchiolitis severity and SNP rs1927911 (TLR4). SNP rs1927911 (TLR4) was recently associated with an increased risk of asthma development in children with a history of bronchiolitis (Lee et al., 2015), so we are following up our patients to verify any association between bronchiolitis severity, the virus type, genetic polymorphisms, and asthma development. Environmental factors have also been shown to interact with the TLR4 genotype to modulate the RSV infection severity (Caballero et al., 2015).

4.2.2. Toll like receptor 2 – TLR2

TLR2 is expressed on the surface of immune cells and tissues as a heterodimer complex with either TLR1 or TLR6. Using knockout mice, TLR2 and TLR6 signaling in leukocytes was shown to activate innate immunity against RSV by promoting tumor necrosis factor alpha, interleukin-6, chemokine (C-C motif) ligand (CCL)2, and CCL5, and was important for controlling viral replication and promoting neutrophil migration and dendritic cell activation in vivo (Murawski et al., 2009). Additionally, TLR4 signaling was reported to influence TLR2 expression following certain stimuli, suggesting a role for both TLR4 and TLR2 in the response to RSV (Fan et al., 2003). One study found no association between TLR1, TLR2, and TLR6 polymorphisms and the bronchiolitis severity (Nuoliivira et al., 2013). We found that death in bronchiolitis is associated with SNP rs1899830 (TLR2) in patients without comorbidities.

4.2.3. Toll like receptor 9 – TLR9

TLR9 has previously been associated with different diseases, such as bronchial asthma (Lazarus et al., 2003). Additionally, RSV was shown to inhibit the production of interferon-γ in human plasmacytoid dendritic cells by TLR9 signaling (Schlender et al., 2005), and TH2 response upregulation, which is characterized seen in severe RSV-associated diseases. Thus, an involvement of TLR9 in the genetics of bronchiolitis seems reasonable. An association of SNP rs5743836 (TLR9) with RSV infection was documented in an earlier study (Mailaparambil et al., 2008), and we found that SNPs rs352162 (TLR9) and rs187084 (TLR9) were associated with a requirement for ICU admission, and that SNP rs352162 (TLR9) was also associated with the need for mechanical ventilation.

4.3. C-C motif chemokine ligand 5 – CCL5

In the course of RSV infection, enhanced chemokine activity modulates cell recruitment and infiltration to the inflammation site. CCL5 is a chemokine produced by CD8+ T-lymphocytes, macrophages, platelets, and epithelial cells that attracts monocytes, eosinophils, basophils, and memory T-lymphocytes to the area of infection. It is highly expressed in respiratory epithelial cell lines, nasal secretions, and broncho-alveolar lavages of RSV-infected subjects. Moreover, evidence supports an association between CCL5 activity and RSV infection (Hattori et al., 2011).

A previous study reported an association between SNPs rs2107538 and rs2280788 in the promoter region and SNP rs2280789 in intron 1 of CCL5 with RSV bronchiolitis (Caballero et al., 2015). However, another study found no association between RSV bronchiolitis and these SNPs when tested separately, but observed a significantly more common combined SNP genotype in patients than in controls (Amanatidou et al., 2008). We found an association between SNP rs2107538*CT (CCL5) and bronchiolitis caused by RSV and RSV subtype A specifically. We also found that SNP rs2107538 (CCL5) was associated with the need for mechanical ventilation in bronchiolitis patients.

4.4. Inducible nitric oxide synthase – NOS2

In the respiratory tract, nitrite oxide (NO) is produced by a wide variety of cell types and is generated via oxidation of l-arginine that is catalyzed by the enzyme NO synthase (NOS). NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO derived from iNOS seems to be a proinflammatory mediator with immunomodulatory effects. In the respiratory tract alone, expression of iNOS has been reported in alveolar type II epithelial cells, lung fibroblasts, airway and vascular smooth muscle cells, airway respiratory epithelial cells, mast cells, endothelial cells, neutrophils, and chondrocytes. The stimuli that cause transcriptional activation of iNOS in these cells varied widely and included endogenous mediators (such as chemokines and cytokines) as well as exogenous factors such as bacterial toxins, virus infection, allergens, environmental pollutants, hypoxia, tumors, etc. The high level of NO released by iNOS has an effect as immune effector molecule in halting viral replication, and in eliminating various pathogens. This mechanism may involve, at least in part, inhibition of DNA synthesis by inactivating of ribonucleotide reductase and by direct deamination of DNA. Finally, NO appears to signal through its reactivity with cysteine groups, particularly those located at consensus motifs for S-nitrosylation with primary sequence or tertiary structure of a protein. One of the general mechanisms of antimicrobial defenses involving NO is S-
nitrosylation by NO of cysteine proteases, which are critical for virulence, or replication of many viruses, bacteria, and parasites (Ricciardolo et al., 2004). A previous study demonstrated an association between SNP rs1060826 (NOS2) and RSV bronchiolitis (Janssen et al., 2007). We found an association between SNP rs1060826 (NOS2) and bronchiolitis caused by rhinovirus.

4.5. Vitamin D receptor – VDR

Vitamin D modulates white blood cell proliferation, maturation, and cytokine expression through the VDR on lymphocytes and macrophages. VDR signaling also contributes to the expression of antimicrobial peptides, which are important for the innate defense against viruses and bacteria (Liu et al., 2007). Lower vitamin D levels have been postulated as a risk factor for respiratory illness based on the well-established seasonality of respiratory infections that occur during winter when UV-B production of vitamin D is low. Subsequent bronchiolitis research in developed countries investigating subclinical vitamin D deficiency supports this hypothesis (Roth et al., 2010). Additionally, a prospective newborn cohort study identified low cord blood vitamin D levels as an independent predictor of RSV infection during the first year of life (Belderbos et al., 2011).

Genetic alterations of VDR have the potential to affect vitamin D signaling through impaired gene transcription, mRNA stability and translation, protein activity, and protein stability. A common VDR SNP, rs2228570, has previously been associated with moderately lower VDR transcriptional activity and a recent meta-analysis concluded that presence of the SNP rs2228570 (VDR) T allele significantly increased the risk of RSV bronchiolitis (McNally et al., 2014). Similarly, we found that SNP rs2228570 (VDR) was associated with death from bronchiolitis in the current study.

In airway epithelial cells, vitamin D controls the expression of signal transducer and activator of transcription (STAT). A recent study demonstrated that the predisposition of SNP rs2228570 (VDR) to severe RSV bronchiolitis may involve the impaired ability of vitamin D to re-strain antiviral signaling in airway epithelia, and that vitamin D fails to regulate STAT1 phosphorylation and downstream gene expression in cells expressing this VDR variant. Strong activation of the STAT1 pathway in RSV-infected cells may therefore contribute to RSV immunopathogenesis (Stoppenburg et al., 2014). Two studies found that another VDR SNP, rs10735810, was associated with an increased likelihood of bronchiolitis and that carriers of the T allele were more likely to develop this disease (Kresfelder et al., 2011; Janssen et al., 2007).

Also regarding genes in the immune system a recent study reported that SNPs rs2227543 (IL-8) and rs2275913 (IL-17) showed significant associations with the severity of acute viral bronchiolitis (Pinto et al., 2017).

Development of an RSV vaccine is a high priority for public health, but attempts to date have been frustrated. Resolving the mechanism by which RSV induces pathogenesis is essential for developing new effective vaccines (Farrag and Almajahi, 2016; Arruviito et al., 2015; Jorquera et al., 2016; Higgins et al., 2016).

4.6. Hardy Weinberg equilibrium

Finally, regarding the HWE, we must remember that the HWE assumes an ideal population, without the interference of evolutionary factors. However, in genes as the involved in immunity, inflammation, and infection controlling, the HWE imbalance may appear secondarily associated with the selection mechanisms that favored a particular allele that can bring a more effective response. The disequilibrium does not invalidate the association study since the groups are part of the same population.

4.7. Study limitations

There are a number of limitations in our study, including: (i) superficial characterization of the population of healthy controls; (ii) because miscegenation in our population is extensive and present in both groups evaluated, the ethnicity assessment was performed in a self-reported manner and we did not study the ancestry, in the controls or in the patients; (iii) sample size reduction due to the non-identification of SNP genotypes for some patients and the difficulty in obtaining the clinical and laboratory markers of all the patients included in the study; (iv) number of evaluations carried out at the same time may lead to confounding; (v) limitations on the size of the sample included in the study and the power of the sample obtained for all the analyzes performed (need for correction by multiple tests with high denominator); (vi) use of candidate genes as a study model, rather than genome-wide association studies, due to technical limitations and high cost involved in the laboratorial analysis.

5. Conclusions

Our findings provide some evidence that genetic variation in selected immune genes may influence the outcomes of severe bronchiolitis but replication in other datasets is needed. The determination of polymorphisms in immune response genes could be used in future work to help predict high-risk infants who might benefit from preventive measures. Knowledge of SNPs associated with severe bronchiolitis will also contribute to an understanding of disease pathogenesis and the innate immune response to its infection. This will be useful in guiding efforts to develop more effective treatment for this potentially fatal infection.

Ethics approval and consent to participate

This study was approved by the National Research Ethics Commission (number: 00869612.7.0000.5404). Legal representatives of patients, and participants of the control group, received explanations about the research and gave written informed consent.

Potential conflicts of interest

The authors declare that they have no conflicts of interest.

Financial disclosure

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Authors’ contributions

AEA conceived and designed this study, selected patients, performed clinical evaluations of patients included in the study, acquired data, analyzed statistical data, drafted, revised, approved and submitted the final manuscript.

FALM conceived and designed this study, performed polymorphism screening, analyzed statistical data, drafted, revised and approved the final manuscript.

CSB conceived and designed this study, performed polymorphism screening, revised and approved the final manuscript.

CWA and JCSB conceived and designed this study, performed viral
identification, revised and approved the final manuscript.

ECB and ATT conceived and designed this study, revised and approved the final manuscript.

MTNR, MBM, CCBA, TO, PGS, EC, MLFM, MCR and JVP selected patients, acquired data, revised and approved the final manuscript.

JDR conceived and designed this study, analyzed statistical data, drafted, revised and approved the final manuscript.

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Appendix A. Supplementary data

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