The present study aimed to determine the role of human parvovirus B19 as an aetiological agent in measles and rubella negative fever/rash patients from Bulgaria between 2004 and 2013. A total of 1,266 sera from all over the country were tested for B19V IgM antibodies and all positives were further investigated by polymerase chain reaction (PCR). Overall, 280 sera (22%) were B19V IgM positive and 227 of these (81%) were also PCR positive. The highest number of IgM positives was found among five to nine year-old children (27%). Eight infected women gave birth to healthy children; one fetus was aborted with hydrops fetalis. Of the 55 genetic sequences obtained, 54 belonged to genotype 1a and one grouped as a genotype 2 outlier. Phylogenetic analysis of all available genotype 2 sequences covering the 994 nucleotide non-structural protein 1 (NS1)/capsid viral protein 1 (VP1) unique region junction, showed that only one other sequence grouped with the outlier strain, forming a clearly distinct and well-supported cluster of genotype 2 (between-group genetic distance: 3.32%). In accordance with B19V nomenclature, this cluster may represent a new subgenotype 2b. The study showed that B19V infections may be falsely identified as rubella or measles in ca 22% of cases, emphasising the need for laboratory confirmation.

Introduction

Human parvovirus B19 (B19V) belongs to the family Parvoviridae, subfamily Parvovirinae, genus Erythrovirus [1]. The viral genome consists of 5,596 nucleotides (nt) encoding among others the non-structural protein 1 (NS1) and the capsid viral protein 1 (VP1). Phylogenetic analysis of a 994 nt fragment of the NS1/VP1 unique region junction (NS1/VP1u) identified three genotypes (1, 2, and 3) of B19V with no clear differences in clinical outcome [2].

B19V infections are associated with different clinical presentations and although typically a mild, self-limiting disease, the infection can cause severe adverse outcomes in certain patients. The main clinical manifestations are erythema infectiosum also known as fifth disease; transient aplastic crisis may occur in individuals with haemoglobinopathies, chronic anaemia in the immunocompromised, acute polyarthralgia syndrome in adults, and sometimes spontaneous abortion and stillbirth after infection during pregnancy [3,4].

There is no vaccine or antiviral drug to prevent B19V infection. In Bulgaria, as well as in most other countries, this infection is not a notifiable disease. However, the clinical presentation of B19V infection is sometimes mistakenly diagnosed as rubella or measles [3-5], which are both notifiable diseases in Bulgaria. An accurate diagnosis of fever/rash illness is necessary for case management and public health control activities [6], in particular in the context of the World Health Organization’s goal to eliminate measles and rubella in the European Region [7,8]. However, in outbreak situations many cases reported as measles or rubella are still not laboratory confirmed in Bulgaria. The laboratory diagnosis of B19V infection in fever (>38.5°C)/rash cases combining serological and molecular methods was introduced in Bulgaria in 2011.

The aim of this mostly retrospective study was to determine the role of B19V as an aetiological agent in measles and rubella negative individuals with fever/rash in...
B19V IgM and/or polymerase chain reaction (PCR) positive gestational age were recorded. In case of childbearing age also pregnancy status and where rash onset and of specimen collection and for women patients, disease diagnosis and complications, date of activity in pregnancy, the pregnancy outcome was monitored. For some patients data on vaccination status available.

For some patients data on vaccination status available. All serum samples were tested for parvovirus B19 IgM antibodies with a commercial indirect enzyme-linked immunosorbent assay (Mikrogen, recomWell Parvovirus Immunosorbent assay (Mikrogen, recomWell Parvovirus). This assay was found to have a high sensitivity of 76.2% and a specificity of 92.5% (Biotrin: 52.4% and 99.5%, respectively) [9]. The assay was performed and interpreted as recommended by the manufacturer and the results were qualitatively categorised as positive, negative or equivocal.

Viral DNA extraction was attempted from all IgM positive serum samples using the NucleoSpin Blood test kits (Macherey-Nagel GmbH & Co. KG). Screening for B9V DNA was performed with primers e1905f and e1987r targeting a region of the NS1 gene (NS1-PCR) [2] and KAPA Taq PCR kits (Kapa Biosystems, Inc.). Positive results were confirmed with a second PCR assay using primers e2717f and e2901r located in the VP1 unique region (VP1u)-PCR [2]. The NS1-PCR has been shown to amplify all three genotypes of B19V and to have a sensitivity threshold of 200 copies of B19V DNA per ml of sample. Sensitivity, specificity and strain distinction of the VP1u-PCR were reported as being similar to those of the NS1-PCR [2]. Viral DNA for sequencing was prepared by nested-PCR amplification of a 1,100-bp region spanning the NS1/VP1u junction [2]. Amplification of additional parts of the B19V genome was attempted for one outlier strain using previously published primers [10]. Each PCR included negative and positive controls. The PCR products were analysed by electrophoresis in 1.5 or 2% agarose gels stained with ethidium bromide. Products for sequencing were purified with the QIAquick PCR purification kits (Qiagen). NS1/VP1u PCR positive samples were sequenced with the BigDye Terminator v3.1 cycle sequencing kit from Life Technologies. Sequences were edited using SeqScape v2.7 and then aligned with references in BioEdit v7.1. The new sequences are available under European Nucleotide Archive accession numbers LN680930–84.

Phylogenetic analyses as well as distance calculations were performed using Molecular Evolutionary Genetics Analysis (MEGA) version 4.0.2 software [11]. For genotyping, phylogenetic trees were constructed based on 994 nt sequences of the NS1/VP1u of human parvovirus B19 [2]. Further analysis of a genotype 2 strain obtained in this study was based on a sequence of 4,070 nt, corresponding to nt positions 845 to 4,914 of the human parvovirus B19 reference sequence (GenBank accession number: NC_000883.2). The Kimura 2-parameter model and the neighbour-joining algorithm of MEGA4 were applied and only bootstrap values ≥70% (1,000 replicates) were considered significant and are shown in the trees.

Results

Epidemiological findings
From 3,872 samples obtained between 2004 and 2013 from individuals with rash/fever, 1,320 were double negative for measles and rubella. Of these, 1,266 with enough leftover material were further tested for B9V IgM antibodies. Most samples investigated were either

Clinical samples

Serum samples from 1,266 measles and rubella IgM-negative patients between one and 47 years of age received at the National Reference Laboratory for Measles, Mumps and Rubella between 2004 and 2013 were included in the study. The sera were from 654 females (52%) and 612 males (48%). Only the first or single samples from measles/rubella suspected patients with fever/rash illness were tested for B19V. The samples were collected as part of measles/rubella surveillance in Bulgaria with the cooperation of regional hospitals and regional public health agencies from all 28 districts in the country (Figure 1). The majority of the samples (n = 1,025, 81%) were from eight districts: Sofia city (n = 396), Blagoevgrad (n = 164), Plovdiv (n = 148), Stara Zagora (n = 81), Varna (n = 92), Rousse (n = 60), Sofia district (n = 43) and Pazardzhik (n = 41) (Figure 1). Besides age, sex and origin of the patients, disease diagnosis and complications, date of rash onset and of specimen collection and for women of childbearing age also pregnancy status and where appropriate gestational age were recorded. In case of B19V IgM and/or polymerase chain reaction (PCR) positivity in pregnancy, the pregnancy outcome was monitored. For some patients data on vaccination status against measles and/or rubella and travel history were available.

Laboratory analysis

All serum samples were tested for parvovirus B19 IgM antibodies with a commercial indirect enzyme-linked immunosorbent assay (Mikrogen, recomWell Parvovirus VP1u PCR positive samples were sequenced with the BigDye Terminator v3.1 cycle sequencing kit from Life Technologies. Sequences were edited using SeqScape v2.7 and then aligned with references in BioEdit v7.1. The new sequences are available under European Nucleotide Archive accession numbers LN680930–84.

Phylogenetic analysis

Phylogenetic analyses as well as distance calculations were performed using Molecular Evolutionary Genetics Analysis (MEGA) version 4.0.2 software [11]. For genotyping, phylogenetic trees were constructed based on 994 nt sequences of the NS1/VP1u of human parvovirus B19 [2]. Further analysis of a genotype 2 strain obtained in this study was based on a sequence of 4,070 nt, corresponding to nt positions 845 to 4,914 of the human parvovirus B19 reference sequence (GenBank accession number: NC_000883.2). The Kimura 2-parameter model and the neighbour-joining algorithm of MEGA4 were applied and only bootstrap values ≥70% (1,000 replicates) were considered significant and are shown in the trees.
collected during a rubella outbreak between 2005 and 2006 (568/1,266; 45%) or during a measles outbreak from 2009 to 2010 (378/1,266; 30%) (Table 1) [12,13].

A total of 280 of the 1,266 patients (22%) were B19V IgM positive (Table 1). The highest percentage of positives was detected in 2013, when more than half of the measles/rubella IgM double negative samples tested were positive for B19V (23/45; 51%), (Table 1). The lowest percentage (7/126; 6%) was observed in 2009 (Table 1). Over the whole study period, the highest number of positive samples were from five to nine year-old children (76/280; 27%) and the lowest from adults over 44 years-old (5/280, 2%, Figure 2). More females (n=162; 58%) than males (42%) were B19V IgM positive – corresponding to 25% (162/654) of all females and 19% (118/612) of all males –, especially among the 25 to 39 year-olds (57 females vs 12 males IgM positive in this age group). Only among five to nine year-old children and 15 to 19 year-olds, were males more affected (Figure 2).

Our cohort comprised 32 pregnant women (aged 20–24 years: 2; 25–29 years: 14; 30–34 years: 11; 35–39 years: 5), nine of whom were B19V IgM and DNA positive (25–29 years: 5; 30–34 years: 3; 35–39 years: 1). While eight of these women gave birth to healthy children, one patient developed complications (hydrops fetalis) and the fetus was aborted.

Overall, 227 of 280 B19V IgM positive samples (81%) were positive in the NS1 PCR, while 81 (29%) and 109 (39%) were positive in the VP1u and NS1/VP1u PCR, respectively (Table 1).

Genotyping and phylogenetic analyses
NS1/VP1u PCR fragments for sequencing and genotyping were available for all years except 2008 (Table 1). While 54 of the 109 NS1/VP1u PCR positive samples had been sequenced and genotyped before (53 times genotype 1a and 1 time genotype 3b) [14], the remaining 55 products were sequenced and genotyped in the present study. All new sequences belonged to genotype 1a, except for one from a female patient in her 30s with complications of reactive arthritis. This sequence grouped as an outlier to the genotype 2 reference sequences (Figure 3). Within genotype 1a, 20 different sequence variants were found (Figure 3). Taking all sequences from Bulgaria from between 2004 until 2013 into account, there was no clear grouping according to the place of origin. All sequences from 2009 (except for the genotype 2 outlier) grouped together as did the sequences from the period between 2010 and 2011, from early 2012 and from 2013. Except for the group formed by 2010 and 2011 sequences, which contained also a strain detected in 2006, all these groups were separated from sequences of other years (Figure 3).

The overall maximum genetic distance was 13.12%. Without the genotype 3b and genotype 2 outlier sequences, this distance was 1.38% (Table 2). The highest maximum and mean genetic distances within genotype 1a sequences were found in 2012 (respectively 1.12% and 0.55%).

Figure 2
Number of human parvovirus B19 IgM positive samples according to age group and sex, Bulgaria, 2004–2013 (n = 280)

Figure 3
Phylogenetic analysis of the Bulgarian human parvovirus B19 sequences identified in this study, 2004–2013

GenBank numbers and provenance of all sequences used in the tree are indicated. All sequences obtained from Bulgaria between 2004 and 2013 are marked with icons according to the year of sample collection. Such sequences are additionally named with location, epidemiological week and year, and the sequences obtained during the present study are highlighted with an asterisk (*). Only one representative strain is shown for identical sequences from the same year and location, separate for previously published and new sequences in this study, and the total number of identical sequences is indicated in brackets. Similar or identical strains from other countries downloaded from GenBank are identified by open circles and reference sequences are identified by accession number, name and genotype.
The sequence from Bulgaria is marked with a black dot and is designated by GenBank number, location, epidemiological week and year. GenBank numbers and provenance of all sequences in the trees are indicated.

A. Analysis of a 994 nt sequence of the Bulgarian strain covering the non-structural protein 1 (NS1)/capsid viral protein 1 (VP1) unique region junction.

B. Analysis of a 4,070 nt sequence of the Bulgarian strain, corresponding to nt positions 845 to 4,914 of the human parvovirus B19 reference sequence (GenBank accession number: NC_000883.2).
Phylogenetic analysis of all genotype 2 sequences covering the 994 nt NS1/VP1u region downloaded from GenBank showed that only one other sequence (BN32.2, DQ333427) grouped with the outlier strain, forming a clearly distinct cluster of genotype 2 (Figure 4a). The within group mean was 1.06% for cluster 1 and 1.65% for cluster 2. The between group distance was 3.32%. Phylogenetic analysis using a 4,070 nt region of B19V confirmed the grouping of the outlier strain with sequence BN32.2 as a clearly separate cluster of genotype 2 (Figure 4b).

Discussion

The present study was based on samples collected during a ten-year period (2004–2013) throughout the country, providing a comprehensive overview of B19V infections during years with widely variable incidences of measles and rubella. Overall, 22% of the fever/rash cases investigated were B19V IgM positive, a relatively high percentage compared with similar studies from e.g. Ireland (4.5%) [3] and England (17%) [15], but lower than the 35.5% reported between 2005 and 2008 from Belarus [16]. Discrepancies between studies may result from the selection of study cohort, design and year(s), measles/rubella control in the country and its geographical location determining the prevalence of different rash/fever causing agents [16-19]. While the lowest yearly proportions of B19V IgM positives in the present study were detected during the 2009 to 2010 measles outbreak (6% and 13%), a high proportion of more than 47% was found towards the end of the 2005 to 2006 rubella outbreak. A recently published study based on a somewhat different patient cohort and only 194 samples collected early during the 2005 to 2006 rubella outbreak, found an even higher rate of IgM positivity of 48.97% [5]. The more similar clinical presentation of rubella and B19V infections, but also the increased B19V incidence reported from elsewhere in Europe in 2005 and 2006 [3,16] may explain the apparent positivity rate difference during the measles and rubella outbreaks. The high rate of B19V IgM positives in 2013 (51%) may be due to a strengthened control of measles and rubella in Bulgaria in light of the 2015 WHO elimination goals in the European Region. Even in the absence of measles and rubella, countries are supposed to analyse samples from suspected cases and to discard at least two suspected cases per 100,000 population per year as non-measles or non-rubella to demonstrate the sensitivity of their surveillance system [8]. With a reduced number of measles and rubella cases, the positive predictive value of positive IgM results decreases. Additional laboratory tests such as viral nucleic acid detection by PCR, IgG titre changes in acute vs convalescent sera or IgG avidity testing may become necessary to confirm IgM test results. Our finding that B19V infections may be falsely identified as rubella or to a lesser extent as measles in ca 22% of the cases is similar to what was reported recently from Belarus [19]. It highlights the benefit of laboratory confirmation of suspected measles/rubella cases, also in outbreak situations.

The highest rate of B19V IgM positivity was found among five to nine year-old children (27%), which is similar to observations from previous studies in

<table>
<thead>
<tr>
<th>Year of sample collection</th>
<th>Proportion of samples from patients with fever and rash, which were negative for both measles and rubella n/N</th>
<th>Number of B19V IgM positives per sera tested n/N (%)</th>
<th>Number of NS1 PCR positives a n</th>
<th>Number of VP1u PCR positives a n</th>
<th>Number of NS1/VP1u PCR positives a n</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>66/93</td>
<td>9/66 (14)</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2005</td>
<td>405/929</td>
<td>89/394 (23)</td>
<td>74</td>
<td>22</td>
<td>52</td>
</tr>
<tr>
<td>2006</td>
<td>182/322</td>
<td>82/174 (47)</td>
<td>70</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td>2007</td>
<td>41/201</td>
<td>9/41 (22)</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2008</td>
<td>42/92</td>
<td>9/42 (21)</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2009</td>
<td>132/610</td>
<td>7/126 (6)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2010</td>
<td>261/1,390</td>
<td>32/252 (13)</td>
<td>27</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>2011</td>
<td>77/106</td>
<td>10/77 (13)</td>
<td>9</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2012</td>
<td>54/56</td>
<td>10/49 (20)</td>
<td>9</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>2013</td>
<td>60/73</td>
<td>23/45 (51)</td>
<td>21</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Total proportions n/N (%)</td>
<td>1,320</td>
<td>3,872 (34)</td>
<td>280/1,266 (22)</td>
<td>227/280 (81)</td>
<td>81/280 (29)</td>
</tr>
</tbody>
</table>

B19V: human parvovirus B19; NS1: non-structural protein 1; NS1/VP1u: NS1/capsid viral protein 1 unique region junction; PCR: polymerase chain reaction; VP1u: capsid viral protein 1 unique region.

a Only B19V IgM positive samples were tested by PCR.

b From the 1,320 samples of patients who tested negative for both measles and rubella, 1,266 had enough material left over for investigating B19V.
Table 2
Genetic distances per year and overall between sequences of human parvovirus B19 detected in Bulgaria, 2004–2013

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of sequences</th>
<th>Maximum genetic distance</th>
<th>Mean genetic distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2005</td>
<td>52 (51)</td>
<td>13.12 (0.56)</td>
<td>0.56 (0.10)</td>
</tr>
<tr>
<td>2006</td>
<td>37</td>
<td>0.61</td>
<td>0.09</td>
</tr>
<tr>
<td>2007</td>
<td>2</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>2009</td>
<td>3 (2)</td>
<td>7.54 (0)</td>
<td>5.03 (0)</td>
</tr>
<tr>
<td>2010</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2011</td>
<td>3</td>
<td>0.10</td>
<td>0.07</td>
</tr>
<tr>
<td>2012</td>
<td>4</td>
<td>1.12</td>
<td>0.55</td>
</tr>
<tr>
<td>2013</td>
<td>6</td>
<td>0.41</td>
<td>0.14</td>
</tr>
<tr>
<td>Overall</td>
<td>109 (107)</td>
<td>13.12 (1.38)</td>
<td>0.56 (0.21)</td>
</tr>
</tbody>
</table>

NA: not applicable.

a In case sequences other than genotype 1a were found in a given year, the values for the genotype 1a sequences only are shown in brackets.

Europe, namely from Belarus [16] and Belgium, England and Wales, Finland, Italy and Poland [18]. More females than males were B19V IgM positive, especially in the 25 to 39 year-olds, possibly because of a high awareness of congenital complications of fever/rash diseases during pregnancy [20,21] and/or because of their closer contacts with children. The PCR targeting the NS1 conserved region detected B19V DNA in more than 81% of IgM positive patients, suggesting that this PCR is of value for the confirmation of B19V infection. Our PCR positivity rate among IgM positives was relatively high compared with the 62% (18/29) reported among rubella and toxoplasmosis suspected but negative patients from Brazil [22] and the 71% (10/14) reported during a B19V outbreak in the Netherlands [23]. Potential explanations for the discrepancies include false positive IgM tests as well as false negative PCR results. The other two PCRs used in our study were less sensitive. At least for the genotyping PCR this may be due to the considerably longer PCR fragment, which is much less likely to be amplified in case of DNA degradation resulting from prolonged sample storage and repeated and long-term defrosting.

The vast majority of the sequences characterised in this study belonged to genotype 1 (53/54, 98%), similar to what has been reported previously from Bulgaria [14]. Although the rate of B19V IgM positivity varied considerably between the years, cases were detected throughout the ten-year period, suggesting an ongoing endemic virus circulation. However, some level of virus clustering by years (in particular during recent years) may indicate repeated virus importations. Genotype 1 is also the most prevalent in most other countries throughout Europe and beyond [14,24-26] and there are recent reports of genotype 3, e.g. from France [2], Greece [14], and the United Kingdom [27]. In contrast, genotype 2 seems to be rare. Infectious B19V genotype 2 variants were identified after organ transplantation [28] and in blood/plasma donations [29,30] in Germany as well as in immunocompromised patients from Poland [31]. A comprehensive study published in 2006 found genotype 2 only in tissues of patients born before 1973 and suggested that this genotype has in principle disappeared from circulation [32]. We found genotype 2 only in one patient born in Bulgaria in the early 1970s and no information about recent travel, transfusion or transplantation history or immunosuppression of the patient was available and no identical strains were found on GenBank. Thus the origin of the strain detected in 2009 remains unclear.

Phylogenetic analysis of all genotype 2 sequences available on GenBank showed that the Bulgarian strain was an outlier forming together with a strain from a German patient (co-infected with genotype 1) [33] a clearly separate cluster of genotype 2 sequences. Both sequences are 98% identical in the 994 nt region and show a between-group genetic distance of 3.32% to the other genotype 2 sequences. Since in addition high bootstrap values are obtained for both clusters and the node linking them, cluster 1 may in accordance with parvovirus B19 nomenclature [34,35] be referred to as subgenotype 2a and cluster 2 as subgenotype 2b.

The introduction of B19V screening of measles and rubella negative specimens from fever/rash patients provided valuable information about the impact of this disease in Bulgaria and will support fever/rash surveillance in the wake of the measles and rubella elimination.

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Conflict of interest
None declared.

Authors’ contributions
SKI was responsible for molecular diagnostic testing, was involved in study coordination, data analysis and interpretation and in writing the first draft of the manuscript; ZGM contributed to study design, data analysis and interpretation and preparation of the first draft of the manuscript; AKT was involved in the serological and molecular diagnostic testing; VPK and LGA were involved in the collection and analysis of clinical and epidemiological data; CPM contributed to data interpretation and revised the manuscript; JMH characterised...
The B 9 viruses, was involved in data analysis and result interpretation as well as the writing of the manuscript.

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5. Moolenaar-de Backer MW, Lukashov VV, van Binnendijk RS, Boot HJ, Zaaijer HL. Global co-existence of two evolutionary lineages of human parvovirus B19, was involved in data analysis and result interpretation as well as the writing of the manuscript.


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