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RESEARCH ARTICLE

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Differential diagnosis of fever and rash cases negative for measles and rubella to complement surveillance activities

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Abstract

In the quest to eliminate measles virus (MV) and rubella virus (Ruv), every suspected case must be properly identified and diagnosed. Since 2017, in Milan (Italy), a total of 978 measles and rubella suspected cases (fever and rash) were investigated and 310 were not laboratory confirmed (discarded cases). To improve surveillance activities, we investigated the presence in discarded cases of 8 other viral pathogens commonly associated with rash: human herpesvirus 6 (HHV-6) and 7 (HHV-7), parvovirus B19 (B19V), enterovirus (EV), Epstein–Barr virus (EBV), human adenovirus (HAdV), cytomegalovirus (HCMV), and SARS-CoV-2. Differential diagnosis was carried out on 289 discarded cases by multiplex real-time PCR assays. At least one pathogen was detected in 188 cases (65.1%) with HHV-7 being the most frequently detected virus. No difference in the number of detected infections overtime was observed and infections were identified in all age groups. As expected, most HHV-6, EV, HAdV, and HCMV-positive cases were found in children aged 0–4 years and HHV-7 was most frequent in the 15–39 age group. In light of the World Health Organization measles elimination goal, the introduction of laboratory methods for differential diagnosis is required for the final classification of clinically compatible cases. The used screening panel allowed us to increase the percentage of virus-positive cases to 87.5%, allowing us to clarify viral involvement and epidemiology, improve diagnosis, and strengthen surveillance activities. As all investigated pathogens were detected, this diagnostic panel was a suitable tool to complement MV and RuV surveillance activities.

KEYWORDS

differential diagnosis, fever and rash, measles, parvovirus B19, rubella, surveillance

Marta Canuti and Antonella Amendola are joint senior authors.

1 | INTRODUCTION

Despite being one of the most contagious diseases known to humankind, with a basic reproductive number between 9 and 18,[1] measles has all the characteristics needed to be eradicated: exclusively interhuman transmission, absence of healthy carriers, and availability of a safe, effective, and easily administered vaccine.[2] Likewise, rubella, a milder and less contagious but of high public health importance viral illness, is an ideal candidate for eradication.[3]

According to the WHO (World Health Organization), measles, rubella, and congenital rubella syndrome elimination can be declared only if a high-quality surveillance system can demonstrate 36 months with no cases of endemic transmission.[4] Despite the noteworthy progress made in implementing surveillance and increasing vaccination coverage with a significant reduction in the burden of these diseases, only the WHO region of the Americas achieved rubella elimination and no WHO region has achieved and sustained measles elimination as of yet.[5–7]

In Italy, the national plan for measles and congenital rubella elimination (PNEMoRc) was approved in 2003.[8] However, the special surveillance system, which included the introduction of laboratory confirmation of measles and rubella cases, to improve surveillance in terms of timeliness, completeness, and comprehensiveness of notifications, was established only in 2007, following the introduction of the "WHO European Region Strategic plan 2005–2010."[3] Further progress was made in 2013 with the introduction of an integrated surveillance network for measles and rubella, which includes molecular and/or serological confirmation for rubella virus (RuV) infection in suspected measles cases that tested negative for measles virus (MV) (discarded cases) and *vice versa*.[8] Additionally, viral genotyping is performed to monitor both viral transmissions and the susceptibility profile of the surveilled population.[9]

As molecular investigation plays a major role in viral spread monitoring, a network for measles and rubella surveillance (MoRoNet-Morbillo e Rosolia network) was established in Italy in 2017.[9] This consists of 15 subnational reference laboratories accredited, coordinated, and supervised by a national reference laboratory in Rome. The Coordinated Research Centre for Epidemiology and Molecular Surveillance of Infections of the University of Milan (EpiSoMI), is one of the two WHO-accredited laboratories in Lombardy, performing MV and RuV surveillance in the Metropolitan City of Milan (a territory of 195 municipalities with a population of about 4 million inhabitants) and surrounding areas.

According to the WHO, a measles clinical case is defined as a person presenting with fever and maculopapular (non-vesicular) rash and at least one of cough, coryza, or conjunctivitis.[10] A rubella clinical compatible case is defined as a person presenting with fever, maculopapular rash and cervical, suboccipital or postauricular adenopathy, or arthralgia/arthritis.[11] However, to improve surveillance system sensitivity and increase the rate of measles and rubella discarded cases,[12] all cases that present with fever and rash should be treated as measles or rubella suspected cases (fever and rash strategy). In Lombardy, all general practitioners and hospital physicians confronting a suspected case send blood or serum and urine and/or oropharyngeal swab samples to accredited laboratories.

Furthermore, in light of the WHO measles elimination goal, the introduction of additional laboratory methods for differential diagnosis is required for reasonably rejecting suspected cases as non-measles and non-rubella, and for the final classification of clinically compatible cases. Indeed, a number of conditions may be associated with fever and maculopapular eruptions in both children and adults, including viral and bacterial illnesses, vasculitis syndromes, and drug reactions.[13,14] Although viral exanthems are usually associated with benign, self-limited diseases, viruses are by far the most common cause of fever and rash.[15] The spectrum of viral causes of exanthems includes human herpesviruses, parvovirus, enteroviruses (EV), adenoviruses, and SARS-CoV-2.[15–17] However, there is a lack of studies that explore the presence of other pathogens associated with morbilliform rash in measles and rubella discarded cases,[18–21] and consequently, little information about the epidemiological characteristics and the burden of each pathogen is available. The aim of this study was to investigate biological samples collected from discarded cases over the years of surveillance activities in Lombardy (2017–2022) to identify other viral pathogens implicated in morbilliform manifestations and improve surveillance activities. Additionally, the epidemiology of the identified viruses was investigated to assess the contribution of each pathogen during the various years and in different age groups.

2 | METHODS

2.1 | Discarded case definition

According to the WHO definition, we considered measles and rubella discarded cases as fever and rash cases that tested negative to both molecular assays aimed to detect MV and RuV nucleic acids in oropharyngeal swabs, blood, or 10–50 mL of urine, and to anti-MV or anti-RuV specific immunoglobulins type M (IgM), investigated in sera collected during the acute phase of infection (1–10 days after the rash onset).[22] Discarded rates were calculated as the number of discarded cases identified over the course of 1 year divided by the average population in the study area (metropolitan area of Milan, about 4 million inhabitants).

2.2 | Specimens

A total of 289 samples collected from as many discarded cases (median age: 19 years old, range: 0–95; 154 male and 135 female subjects) between March 2017 and December 2022 were investigated: 78 were collected in 2017, 74 in 2018, 104 in 2019, 11 in 2020, 18 in 2021, and 4 in 2022. For each case, only the urine sample or the oropharyngeal swab was tested according to sample availability. The differential diagnosis was performed on nucleic acids isolated during previous surveillance activities with the NucliSENS®

easyMAG™ automated system (bioMérieux bv) from 0.2 to 1 mL of UTM® (Copan) ($N = 228$) or using pellet obtained from 1.5 to 50 mL of urine ($N = 61$) as input and stored at −80°C in the biobank of the Laboratory at the University of Milan.[23]

2.3 | Differential diagnosis

Two commercial TaqMan-Based Multiplex Real-Time PCR assays (Siemens Healthineers) were used to identify the nucleic acids of seven viral pathogens: human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), parvovirus B19 (B19V), EV, Epstein–Barr virus (EBV), human adenovirus (HAdV), and human cytomegalovirus (HCMV). Amplification reactions were performed following the manufacturer's instruction using 10 µL of isolated nucleic acid per reaction (fever and rash mix 1—HHV-6, HHV-7, and B19V, fever and rash mix 2—EV, and ACE mix—EBV, HAdV, and HCMV). Real-Time PCRs were performed on QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™; Thermo Fisher Scientific). Samples collected from 2021 onwards were also tested for SARS-CoV-2 according to the CDC protocol.[24] Each run included positive controls provided with the amplification kit (plasmids) and negative controls (nuclease-free water). An assay was determined as positive when the cycle threshold growth curves crossed the threshold line within 40.00 cycles.

Additionally, as results about the presence of SARS-CoV-2 in older samples were available from previous investigations,[14] information about this pathogen was also included in our analyses. Four samples that previously tested positive for SARS-CoV-2 were not tested for other pathogens as the sample was no longer available.[25]

2.4 | Data analyses

Comparisons of proportion were accomplished by the χ^2 test and p-values < 0.05 were considered statistically significant. Analyses were conducted using the OpenEpi online tool.[26]

Age groups were defined according to the recommendations of the Istituto Superiore di Sanità on measles and rubella surveillance[27]: 0–4 years ($N = 101$), 5–14 years ($N = 24$), 15–39 years ($N = 94$), 40–64 years ($N = 50$), and >64 years ($N = 20$). The network plot was built with Past 4.08.[28]

3 | RESULTS

3.1 | Evaluation of discarded cases

From the beginning of surveillance activities (March 2017) to December 2022, we investigated 978 cases of suspected measles and/or rubella, 668 of which were confirmed as measles (68.3%). No cases tested positive for rubella. This corresponded to a rate of discarded cases, calculated with all received samples (including both officially notified and non-notified cases), between 0.2 (2022) and 2.9 (2019) per 100 000 population. Figure 1 shows the percentage and the rates of measles and non-measles and non-rubella cases over the years.

3.2 | Identified viruses and multiple infections

After testing MV/RuV-negative cases for a panel of viruses capable of causing rash, we found evidence of viral infections in 188 out of the 289 investigated specimens (65.1%), leaving only 12.5% (122/978) of the total number of suspected cases without a viral diagnosis. Infections were equally distributed between males and females ($p = 0.8$) and positivity rates were not significantly different between the various age classes (56.0% −71.3%, $p = 0.5$) (Table 1). The overall positivity rate was significantly lower in urine (22/61, 36.1%) than in swabs (166/228, 72.8%, $p < 0.000001$) (Table 1).

HHV-7 was the most frequently detected pathogen (32.2%, 95% CI: 25.5–38.9), followed by EBV (28%, 95% CI: 21.8–34.3), HHV-6 (11.8%, 95% CI: 7.7–15.8), HAdV (6.9%, 95% CI: 3.8–10.0), B19V (5.9%, 95% CI: 3.0–8.7), HCMV (4.2%, 95% CI: 1.8–6.5), SARS-CoV-2 (3.5%, 95% CI: 1.3–5.6), and EV (2.8%, 95% CI: 0.8–4.7) (Supporting Information: Figure S1). EVs were only identified in swabs, while all other pathogens were detected in both urine and respiratory samples (Table 1).

Overall, 60.1% (113/188) of the positive samples contained a single pathogen, while the viral genome of at least two pathogens (multiple infections) was detected in 39.9% (75/188) of the specimens. Particularly, infections sustained by two, three, or four pathogens were detected in 64, 10, and 1 cases, respectively (Supporting Information: Table S1). The most frequent multiple infection was HHV-7/EBV double infection (Supporting Information: Table S1), likely reflecting viral distribution trends observed in our population (Supporting Information: Figure S1).

The multiple infection rate (proportion of multiple infections over the total number of identified cases for one pathogen) was different for the various investigated viruses (Figure 2A): samples positive for B19V and HMCV showed the highest multiple infection rates (approximately 83%) while this was the lowest (<40%) for EV and was ~53-62% for the other viruses. Interestingly, 75% (9/12) of HCMV multiple infections involved EBV and only 1 patient that tested positive for EVs was also positive for a herpesvirus (HHV-6) (Figure 2B). In fact, in 71 out of 75 cases (94.7%), multiple infections involved viruses belonging to the viral family *Herpesviridae* (HHV-6, HHV-7, EBV, and/or HCMV).

3.3 | Seasonality and age distribution

Throughout the studied period, several peaks of suspected cases were observed, with the three biggest ones recorded in spring 2017, 2018, and 2019. These all corresponded to MV outbreaks and to an

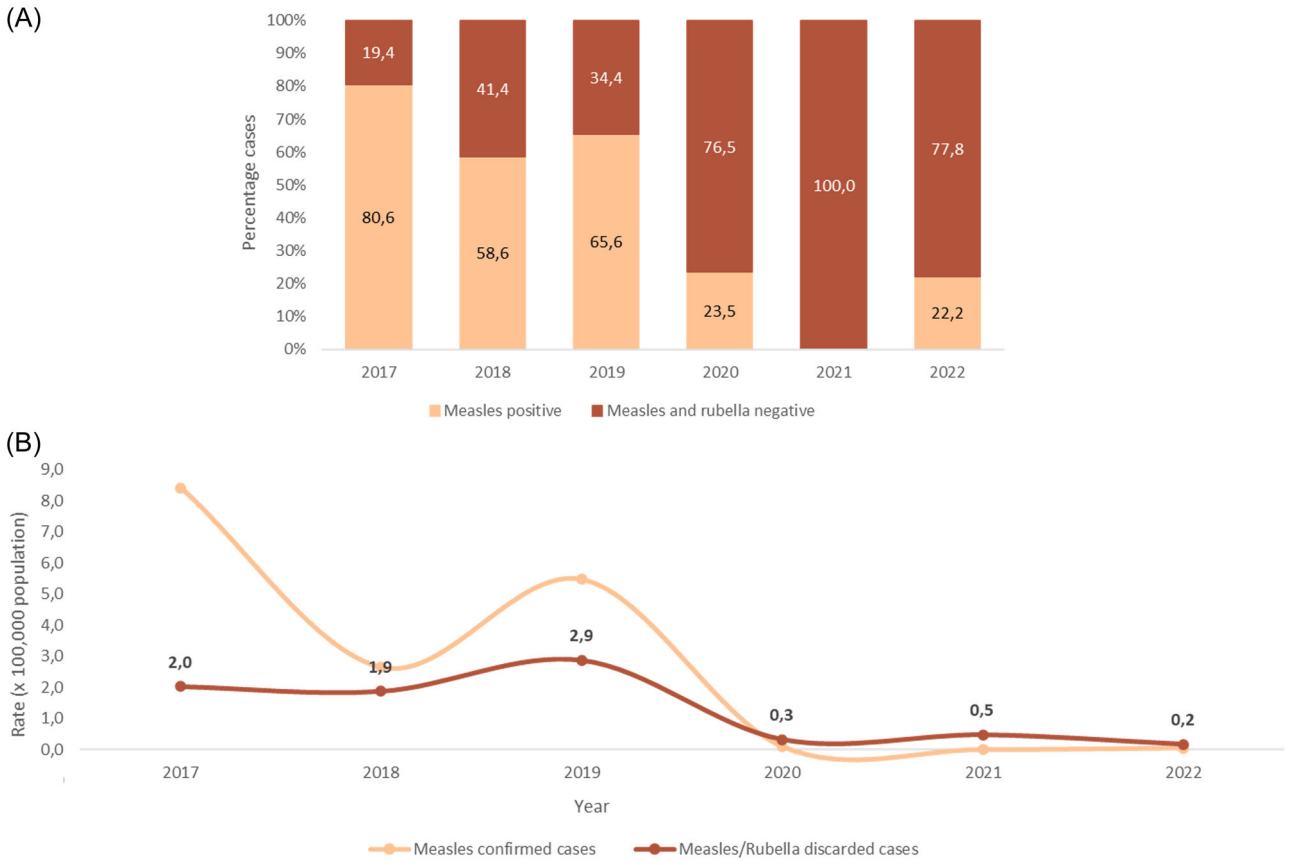

FIGURE 1 Proportion (A) and rate (B) of measles cases and measles and rubella discarded cases over the 6 years of surveillance activities (2017–2022).

increase in positivity for HHV-6 and HHV-7 (Supporting Information: Figure S2A and B).

The quote of discarded cases that tested positive for at least one of the investigated viruses was 55.1% in 2017 (43/78), 52.7% in 2018 (39/74), 78.8% in 2019 (82/104), 63.3% in 2020 (7/11), 77.8% in 2021 (14/18), and 75% in 2022 (3/4) (Table 1), leaving an overall percentage of undiagnosed cases of 9.1% in 2017 (38/418), 19.9% in 2018 (36/181), 9.9% in 2019 (33/334), 35.3% (6/17) in 2020, 26.3% in 2021 (5/19), and 44.4% (4/9) in 2022. While the prevalence of herpesviruses fluctuated during the years, the frequency of B19V increased in the years of low (2020–2022) MV circulation (13/256: 5.1% vs. 4/13: 12.1%) although this difference was not significant, possibly because the number of investigated samples since 2020 was low. No particular seasonal trend was observed for any of the viruses (Supporting Information: Figure S2C).

While HHV-6, EV, HAdV, and HCMV infections were mostly identified in children under 4 years of age (58.8%, 87.5%, 50.0%, and 66.7% of positive samples, respectively), HHV-7 infection was detected mainly in young adults (55.9% considering all infections, 68.2% considering only single infections). B19V, EBV, and SARS-CoV-2 were more equally distributed among younger patients and adults, with a prevalence ranging from 3.2% to 10%, 25.5%–33.3%, and 0%–6%, respectively (Figure 3 and Table 1). EBV was the most prevalent virus in younger individuals (0–14 years), while HHV-7 was the most prevalent virus in all other age groups (Table 1). HHV-6 and HCMV were more prevalent in young (0–14 years) and elderly but less prevalent in other age groups; EV was found almost exclusively in young kids (0–4 years); the prevalence of HAdV was higher in the two youngest age groups (0–14 years); the one of HHV-7 was higher in patients >4 years; the prevalence of EBV, B19V, and SARS-CoV-2 was constant across ages (Table 1). Finally, multiple infection rates varied across ages, being 33.3% in children aged 0–4 years, 60.0% in children aged 5–14 years, 34.3% in younger adults (15–39 years), 50.0% in adults (40–64 years), and 58.3% in the elderly (>64 years).

4 | DISCUSSION

In Italy, endemic transmission of measles is still ongoing, although the last epidemic wave peaked in April 2019. Since then, the number of both suspected and laboratory-confirmed measles cases abruptly decreased (from 27 per million population to 0.3 per million population).[27] A similar decline in the number of cases has been observed in other countries and this could be due to under-reporting and under-diagnosis or be a real decrease due to COVID-19 pandemic containment measures.[29] An analogous trend was observed in Italy for rubella, which peaked in 2017 (1.2 cases per million) and then decreased to 0.1 cases per million in 2022.[27]

TABLE 1 Number (percentage) of samples that tested positive for the viruses investigated in this study stratified by age, sex, year, sample type, and infection type.

	HHV-6 *N* (%)	HHV-7 *N* (%)	EBV *N* (%)	HCMV *N* (%)	Any herpesvirus *N* (%)	B19V *N* (%)	EV *N* (%)	HAdV *N* (%)	SARS-CoV-2 *N* (%)	At least one virus *N* (%)
Total population (*N* = 289)	34 (11.8)	93 (32.2)	81 (28.0)	12 (4.2)	163 (56.4)	17 (5.9)	8 (2.8)	20 (6.9)	10 (3.5)	188 (65.1)
Male (*N* = 154)	20 (13.0)	47 (30.5)	44 (28.6)	5 (3.2)	86 (55.8)	10 (6.5)	5 (3.2)	7 (4.5)	6 (3.9)	101 (65.6)
Female (*N* = 135)	14 (10.4)	46 (34.1)	37 (27.4)	7 (5.2)	77 (57.0)	7 (5.2)	3 (2.2)	13 (9.6)	4 (3.0)	87 (64.4)
Oropharyngeal swab (*N* = 228)	28 (12.3)	**90 (39.5)**	69 (30.3)	**3 (1.3)**	**144 (63.2)**	15 (6.6)	8 (3.5)	69 (30.3)	16 (0.07)	**144 (63.2)**
Urine sample (*N* = 61)	6 (9.8)	**3 (5)**	12 (19.7)	**9 (14.8)**	**19 (31.1)**	2 (3.3)	0	12 (19.7)	4 (6.6)	**19 (31.1)**
Single infection (*N* = 289)	15 (5.2)	44 (15.2)	31 (10.7)	2 (0.7)	92 (31.8)	3 (1.0)	5 (1.7)	9 (3.1)	4 (1.4)	113 (39.1)
Double infections (*N* = 289)	13 (4.5)	41 (14.2)	39 (13.5)	7 (2.4)	60 (20.8)	12 (4.2)	3 (1.0)	8 (2.8)	5 (1.7)	64 (22.1)
Triple infections (*N* = 289)	5 (1.7)	7 (2.4)	10 (3.5)	3 (1.0)	10 (3.5)	2 (0.7)	0 (0.0)	2 (0.7)	1 (0.3)	10 (3.5)
Quadruple infections (*N* = 289)	1 (0.3)	1 (0.3)	1 (0.3)	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	1 (0.3)	0 (0.0)	1 (0.3)
2017 (*N* = 78)	5 (6.4)	20 (25.6)	25 (32.1)	5 (6.4)	**39 (50.0)**	3 (3.8)	1 (1.3)	4 (5.1)	**0 (0.0)**	**43 (55.1)**
2018 (*N* = 74)	8 (10.8)	19 (25.7)	14 (18.9)	2 (2.7)	**31 (41.9)**	4 (5.4)	5 (6.8)	5 (6.8)	**0 (0.0)**	**39 (52.7)**
2019 (*N* = 104)	16 (15.4)	45 (43.3)	33 (31.7)	2 (1.9)	**74 (71.2)**	6 (5.8)	2 (1.9)	10 (9.6)	**5 (4.8)**	**82 (78.8)**
2020 (*N* = 11)	1 (9.1)	2 (18.2)	2 (18.2)	1 (9.1)	**4 (36.4)**	1 (9.1)	0 (0.0)	1 (9.1)	**2 (18.2)**	**7 (63.6)**
2021 (*N* = 18)	2 (11.1)	5 (27.8)	6 (33.3)	2 (11.1)	**12 (66.7)**	3 (16.7)	0 (0.0)	0 (0.0)	**3 (16.7)**	**14 (77.8)**
2022 (*N* = 4)	2 (50.0)	2 (50.0)	1 (25.0)	0 (0.0)	**3 (75.0)**	0 (0.0)	0 (0.0)	0 (0.0)	**0 (0.0)**	**3 (75.0)**
0–4 years (*N* = 101)	**20 (19.8)**	**9 (8.9)**	28 (27.7)	**8 (7.9)**	52 (51.5)	6 (5.9)	**7 (6.9)**	10 (9.9)	4 (4.0)	66 (65.3)
5–14 years (*N* = 24)	**3 (12.5)**	**7 (29.2)**	8 (33.3)	**2 (8.3)**	13 (54.2)	2 (8.3)	**0 (0.0)**	3 (12.5)	1 (4.2)	15 (62.5)
15–39 years (*N* = 94)	**5 (5.3)**	**52 (55.3)**	24 (25.5)	**0 (0.0)**	63 (67.0)	3 (3.2)	**1 (1.1)**	5 (5.3)	2 (2.1)	67 (71.3)
40–64 years (*N* = 50)	**3 (6.0)**	**17 (34.0)**	15 (30.0)	**0 (0.0)**	23 (46.0)	5 (10.0)	**0 (0.0)**	1 (2.0)	3 (6.0)	28 (56.0)
>64 years (*N* = 20)	**3 (15.0)**	**8 (40.0)**	6 (30.0)	**2 (10.0)**	12 (60.0)	1 (5.0)	**0 (0.0)**	1 (5.0)	0 (0.0)	12 (60.0)

Note: Statistically different percentages are in bold.

Abbreviations: B19V, parvovirus B19; EBV, Epstein–Barr virus; EV, enterovirus; HAdV, human adenovirus; HCMV, human cytomegalovirus; HHV-6, human herpesvirus-6; HHV-7, human herpesvirus-7; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Simultaneously, a decrease in routine childhood vaccination rates has been reported. In Italy, vaccination uptake for the second dose of measles-containing vaccines declined by two percentage points compared to 2019 (85.6% in 2021 vs. 87.6% in 2019).[30] Pandemic-related disruptions, together with the lifting of non-pharmaceutical interventions to prevent COVID-19, created what UNICEF and WHO called the "perfect storm," which is likely going to increase the chance of measles resurgence in the near future.[31]

During the surveillance activities, we observed three peaks in measles cases: in March to June 2017 (337 cases), coinciding with the beginning of surveillance activities, in April to June 2018 (61 cases), and in the first 7 months of 2019 (217 cases). Since August 2019, a consistent decrease in measles cases has been observed, with only 8 confirmed cases between September 2019 and December 2022, none of which were autochthonous. We did not observe rubella during the whole study period.

In the quest to eliminate measles and rubella, every suspected case must be properly identified and diagnosed. Since several pathogens share the ability to cause morbilliform rash, the differentiation of fever and rash cases by clinical symptoms is difficult. Therefore, the implementation of diagnostic tools able to recognize different etiological agents is essential for the final classification of clinically compatible cases. Differential diagnosis allowed us to reduce the number of undiagnosed cases from 32% to 12% (including 21 cases untested due to sample unavailability), corresponding to an overall percentage of viral identification of 88%.

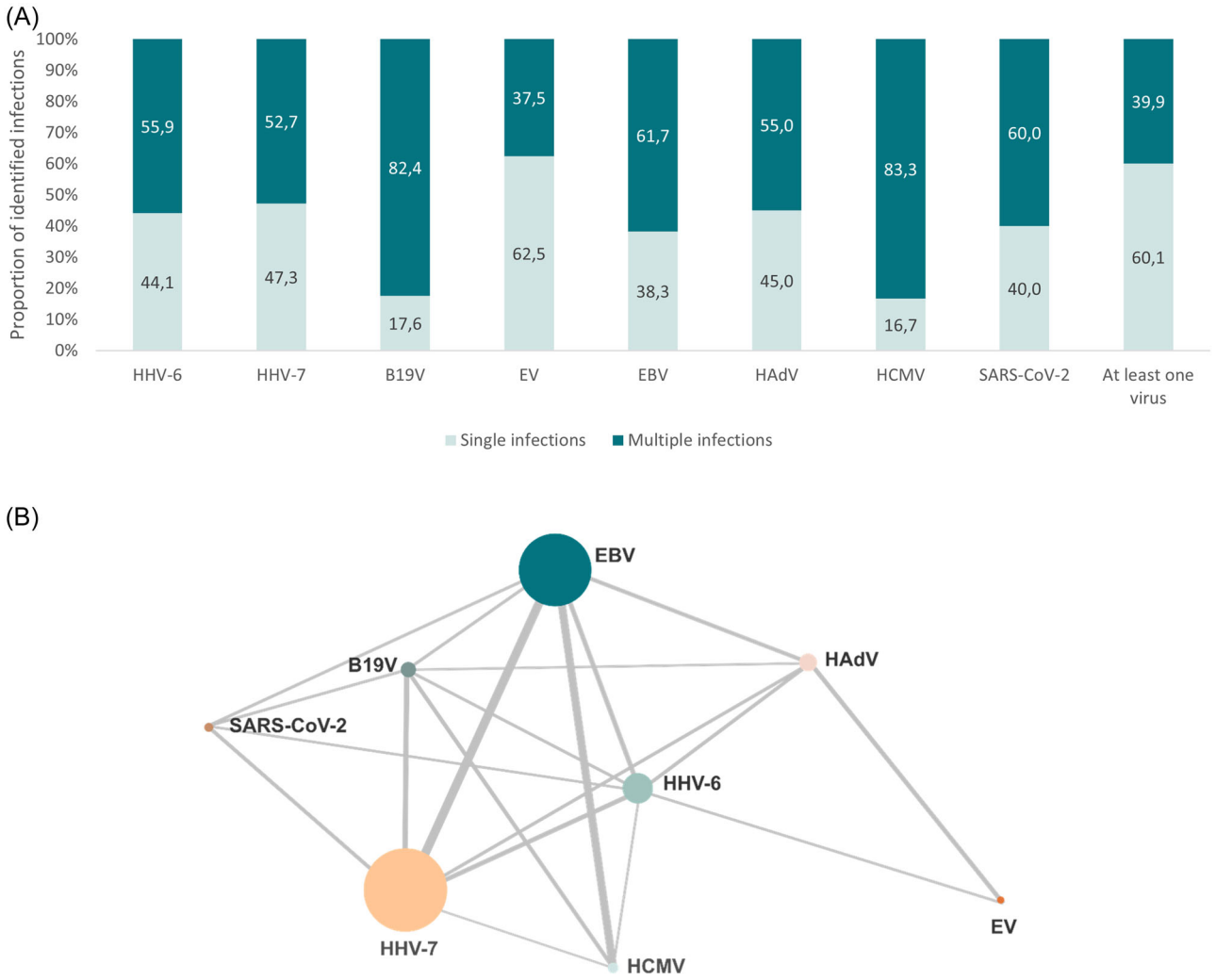

FIGURE 2 Multiple infections identified in this study. Proportions of single and multiple infections for each identified pathogen (A) and network plot of multiple infections (B). In the plot, the size of the circles is proportional to the number of positive patients and the thickness of the line is proportional to the number of detected multiple infections. B19V, parvovirus B19; EBV, Epstein−Barr virus; EV, enterovirus; HAdV, human adenovirus; HCMV, human cytomegalovirus; HHV- 6, human herpesvirus-6; HHV-7, human herpesvirus-7; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

This percentage is higher compared with other studies performing fever and rash differential diagnosis (e.g., 52.7% in a study conducted in Belarus,[20] 11.2% in a study conducted in Catalonia,[18] and 16.1% in Cuba[32]). However, the number of investigated pathogens was higher in our study, and this may explain some of these differences. Moreover, these studies were conducted in countries close to measles and rubella elimination[19,20] or that already achieved the elimination goal.[18,32]

To the best of our knowledge, our study is the first to extend fever and rash differential diagnosis to 10 pathogens using molecular methods. With molecular screening, we identified at least one pathogen in 65% of the tested samples. With a positivity rate of 32%, HHV-7 was the most frequently detected pathogen, followed by two other members of the family *Herpesviridae*, EBV and HHV-6. Although at lower frequencies (3%−7%), we also observed the presence of the other investigated pathogens: HAdV, B19V, HCMV, EV, and SARS-CoV-2. These results suggest that this diagnostic panel targeting eight additional viruses is a suitable tool to complement MV and RuV surveillance activities.

Our data differ from other studies in which B19V was the most frequently investigated and detected pathogen in fever and rash cases.[20,32,33] However, the assessment of virus distribution among individuals with these clinical manifestations varies widely in literature according to considered years, geographical area, study design, and laboratory approach. Nonetheless, during the last years of low MV circulation, we observed an increase in B19V cases. Since there were only a few samples available from these years and considering that this increase was not statistically significant, future surveillance activities should confirm whether this trend continues.

The range of viral causes of exanthems has widened due to the continuous emergence of new viruses and new diagnostic methods. For example, cutaneous manifestations, particularly morbilliform

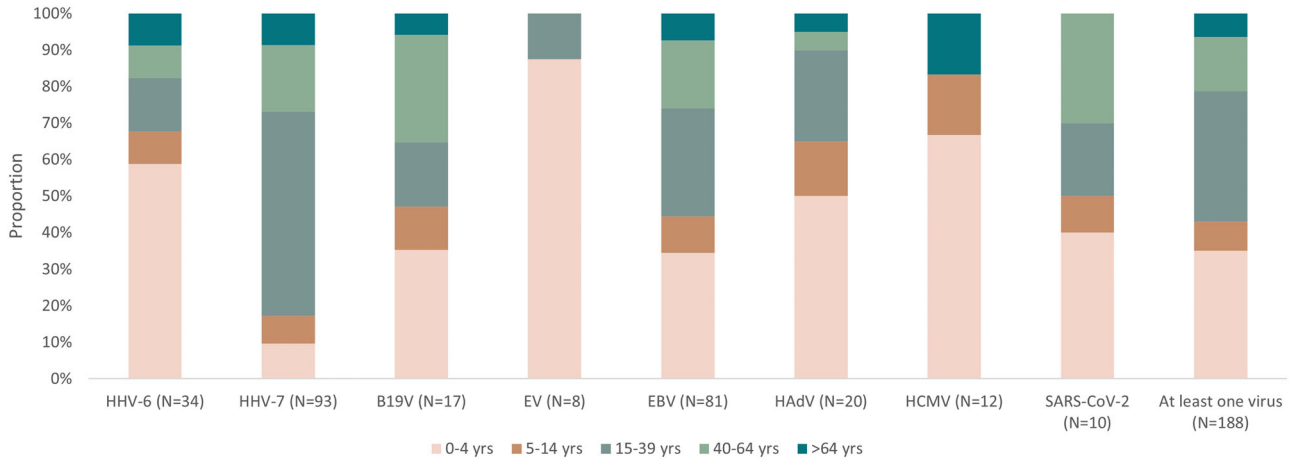

FIGURE 3 Age distribution of positive patients for each virus. B19V, parvovirus B19; EBV, Epstein–Barr virus; EV, enterovirus; HAdV, human adenovirus; HCMV, human cytomegalovirus; HHV- 6, human herpesvirus-6; HHV-7, human herpesvirus-7; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

rash,[34] have been reported in patients with SARS-CoV-2 infection since late 2019.[25,35,36] In our study, we identified SARS-CoV-2 in 5% −18% of the investigated samples, depending on the considered year, and these results underline the importance of including this pathogen in fever and rash differential diagnosis, especially now that SARS-CoV-2 diagnostic is no longer routinely performed.

For none of the investigated pathogens, a seasonal distribution was found, and infections were identified in all age groups. As expected, most HHV-6, EV, HAdV, and HCMV-positive cases were found among children aged 0–4 years.[19,20,32] On the other hand, we detected most HHV-7 infections in the 15–39 years age cohort, even though literature data report that primary infection mostly occurs during early childhood.[37] The prevalence of the other investigated pathogens was not affected by age, however, the percentage of samples that tested positive for at least one virus was high in all age groups (56%−71%), indicating that this differential diagnostic can be useful in clarifying fever and rash cases in all ages. Forty percent of investigated cases showed the presence of multiple infections, most of which involved two pathogens; however, one case of quadruple infection was also detected. Notably, B19V and HCMV, the two viruses with the lowest positivity rates, were also those with the highest multiple infection rates, consistent with other studies that reported frequent multiple infections for these two pathogens, especially with members of the family *Herpesviridae*.[38–40] Nevertheless, although a number of studies investigated the presence of B19V in fever and rash cases,[20,32] to the best of our knowledge, there are no studies that included HCMV in differential diagnostic panels. Interestingly, three quarters of HCMV multiple infections involved EBV and the significance of this finding should be assessed in future studies.

Although the pathogens investigated in this study are known to cause cutaneous manifestations, they are also all found in healthy individuals. Notably, 56% of the investigated samples tested positive for at least one human herpesvirus. Following primary infection, usually asymptomatic and clinically unrecognized, herpesviruses are able to establish a life-long latent infection and they are likely to be reactivated as a consequence of a variety of conditions (e.g., local trauma, surgery, systemic stress, fever, UV-exposure, emotional stress, hormonal changes), especially in immunocompromised individuals. They are also ubiquitously present and may not always be responsible for causing all symptoms, especially considering their opportunistic nature.[41,42] Additionally, as the herpesviruses investigated in our study are mainly excreted in the saliva,[43] oropharyngeal swabs may not represent the optimal sample to assess HHV association with symptoms. Nonetheless, all studied herpesviruses were also found in urine. Furthermore, in a proportion of cases, herpesviruses may have been the (co-)responsible agents causing rash, but only as a result of a reactivation induced by other causes, particularly other pathogens. Indeed, late primary infection with members of the family *Herpesviridae* seems to be unusual and reactivation can explain the peculiar HHV-7 infection peak we found in older individuals.[37,44,45] Furthermore, several studies indicated the potential role of SARS-CoV-2 infection in the reactivation of human herpesviruses in latently infected host cells.[46,47] While further research is needed to fully clarify the association between coinfections and skin rash in COVID-19 patients, our observation that 60% of SARS-CoV-2-positive patients were also positive to one or more herpesviruses could be explained by herpesviral reactivation.

Finally, it has to be considered that only a subset of HAdV and EV are known to cause rash[19] and the assay we employed was not capable of discriminating between different types. This could mean that all or some of the HAdV and EV we observed were not the actual cause of the rash; this is especially true for those identified in respiratory samples since these two pathogens are often found in oral environments.[14] Future studies should, therefore, be focused on molecularly typing these viruses to assess which types are identifiable in these cases and what is the proportion of rash-associated types. Everything considered, future studies should also assess the prevalence of the investigated viruses also in MV-positive patients and in rash-free individuals so that stronger conclusions about their role in causing rash in various populations can be drawn.

One limitation of our study is that the differential diagnosis was not exhaustive. Indeed, other viruses (e.g., Dengue virus types 1–4, West Nile virus, Zika virus) and bacteria (e.g., *Streptococcus pyogenes* and *Mycoplasma pneumoniae*) can be detected in patients with a skin rash similar to a morbilliform eruption.[48] Additionally, the morbilliform rash can be associated with noninfectious diseases and reactions to drugs, such as sulfonamide.[49] Furthermore, it is well known that viruses may act as cofactors in promoting skin rashes induced by drugs, and, especially in children, most of these reactions involve herpesvirus acute infections or reactivations.[50] Unfortunately, in our study data regarding antibiotic administration were not available and we were not able to further investigate the association between adverse drug reaction and viral infections. Additionally, since investigated pathogens may have different diagnostic windows and may reach different viral loads in the respiratory tract or urine, further studies including more samples collected over a larger period of time can be considered.

In conclusion, our study allowed us to reduce the percentage of undiagnosed cases of fever and rash, allowing us to clarify viral involvement, improve diagnosis, and strengthen surveillance activities. Since a small proportion of cases still remained unexplained and some instances of rash could have been a consequence of herpesviral reactivation, further studies are still required to clarify the role of the investigated pathogens in fever and rash cases, especially considering the paucity of studies available on this topic. It is important to keep investigating non-MV and non-RuV fever and rash cases not only to expand our knowledge about the epidemiology of rash-associated viruses and their clinical manifestations, but also to improve surveillance quality and monitor properly all cases as we progress toward measles and rubella elimination. Indeed, the trend of discarded cases we observed during the last years of investigation reflects the expected scenario in a country reaching measles and rubella elimination, with a decrease in measles and rubella incidence and an increase in the proportion of discarded cases, in which other pathogens may be involved. Elimination is also a question of quality and sensitivity of surveillance, and on the path toward measles elimination, it is essential to support the rejection of measles and rubella-negative cases with differential diagnosis.

AUTHOR CONTRIBUTIONS

Antonella Amendola developed the idea and designed the study. Clara Fappani, Maria Gori, Mara Terraneo, Erica Bilardi, and Daniela Colzani conducted experiments, and collected and analyzed data. Clara Fappani, Maria Gori, and Marta Canuti created graphs and tables. Clara Fappani, Maria Gori, and Marta Canuti wrote the manuscript. Silvia Bianchi, Elisabetta Tanzi, and Antonella Amendola critically revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article.

ETHICS STATEMENT

Ethical review and approval and patient consent were waived for this study as it was carried out as part of the Integrated Measles-Rubella Surveillance, performed by law in accordance with the Prime Minister's Decree of 3 March 2017 (https://www.gazzettaufficiale.it/eli/id/2017/05/12/17A03142/sg accessed on 3 April 2023).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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