Diagnostic Microbiology & Infectious Disease 112 (2025) 116754

Contents lists available at ScienceDirect

Diagnostic Microbiology & Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

The frequency of SARS-CoV-2 in stool: A prospective study of healthy preschool children in Sweden

Anna Heydecke [a,b,*], Susanne Sütterlin [c], Åsa Melhus [b,1], Karolina Gullsby [a,1]

[a] Centre for Research and Development, Uppsala University, Region Gävleborg, Gävle, Sweden
[b] Department of Medical Sciences, Section of Clinical Microbiology, Uppsala University, Uppsala, Sweden
[c] Department of Women's and Children's Health, Pediatric Inflammation, Metabolism and Child Health Research, Uppsala University, Uppsala, Sweden

ARTICLE INFO

Keywords:
COVID-19
Epidemiology
Omicron
Transmission sources

ABSTRACT

The study investigated the frequency of SARS-CoV-2 among healthy preschool children in two Swedish municipalities, a country where preschools remained uniquely opened throughout the pandemic.

Stool samples were obtained from diapers collected at preschools in November-December 2021 and analyzed for SARS-CoV-2 using real-time reverse transcriptase polymerase chain reaction. Additionally, laboratory data concerning COVID-19 in the two regions were reviewed.

Sixty-seven percent of invited preschools participated, providing 350 stool samples (Uppsala n=231, Gävle n=119). The children had a median age of 24 months (range 12-72). SARS-CoV-2 was not detected in any stool sample during the study period, which coincided with the initial omicron wave. The first pediatric omicron case appeared nearly two weeks after the first adult case.

These findings suggest preschool children did not constitute a silent source nor played a major role in the transmission of SARS-CoV-2 during the first phases of the largest wave of COVID-19, so far.

1. Introduction

In late 2019, a cluster of severe pneumonia cases was observed in Wuhan, China [1]. The causative agent was a previously unknown coronavirus, referred to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The illness caused by SARS-CoV-2 was then recognized as coronavirus disease 2019 (COVID-19). It disseminated rapidly and was declared a pandemic by the World Health Organization in March 2020 [2]. On May 5 2023, the same organization declared COVID-19 no longer a global health emergency [3], though it continues to pose a risk, especially to older adults with weakened immunity.

SARS-CoV-2 causes a wide range of symptoms, from mild to severe. Although COVID-19 is predominantly considered a respiratory disease, gastrointestinal manifestations, such as diarrhea, abdominal pain, vomiting and nausea are not uncommon [4]. Children tend to develop mainly a mild form with symptoms typical of acute respiratory tract infection [5]. Compared to adults, children are less likely to be hospitalized and the mortality rate is low [5,6], but serious complications, e.g. multisystem inflammatory syndrome in children, can occur [7]. Since a considerable number of SARS-CoV-2 infections in children are asymptomatic [8] and young children may have difficulties communicating subtle symptoms, infections are at risk of going undiagnosed in the younger age groups.

Transmission of SARS-CoV-2 predominantly occurs by inhalation of respiratory droplets [9]. Though infectious SARS-CoV-2 has been isolated from feces of COVID-19 patients [10], and viral shedding in stool may continue for several weeks, even after the respiratory samples are no longer positive [11,12]. Questions have therefore been raised about whether feces and diapers could act as a source of transmission.

When the COVID-19 epidemic emerged, many countries recommended extensive screening programs to prevent the spread of SARS-CoV-2 into the community. Children were largely exempted from these recommendations and they comprised a minority of all confirmed cases [13]. Swedish children up to 6 years of age were not covered by the national screening recommendations. Due to the low screening rate and high degree of asymptomatic and mild infections in children, limited data on the burden of COVID-19 in the younger pediatric population are available. Furthermore, preschool children have so far not been covered by SARS-CoV-2 vaccination. Children may thereby constitute a population who is relatively susceptible to SARS-CoV-2 and act as a silent

* Corresponding author at: Department of Clinical Microbiology, Region Gävleborg, SE-801 87, Gävle, Sweden.
E-mail address: anna.heydecke@regiongavleborg.se (A. Heydecke).

[1] Contributed equally as co-senior authors.

https://doi.org/10.1016/j.diagmicrobio.2025.116754
Received 5 December 2024; Received in revised form 17 February 2025; Accepted 17 February 2025
Available online 26 February 2025
0732-8893/

reservoir. This group is especially interesting, since preschools and schools for primary education in Sweden remained uniquely open during the whole pandemic.

The aims of this prospective study were to estimate the frequency of SARS-CoV-2 in stool in healthy Swedish preschool children during the initial phases of a new wave of COVID-19 in order to gain better understanding of the SARS-CoV-2 epidemiology in young children, and to investigate whether this age group may constitute a silent reservoir of SARS-CoV-2.

2. Material and methods

2.1. Settings and ethics

This prospective study was conducted in Uppsala during calendar weeks 45-48 and in Gävle during calendar weeks 49-50 2021. This year, 13,183 preschool children had their residency in Uppsala and 5,784 in Gävle, two municipalities in Sweden. Eighty-two percent of the children living in Uppsala attended preschools, and of them 65% attended municipal preschools. The corresponding figures for Gävle were 87% and 92%, respectively [14]. A majority of the children spent 6–8 h/workday in the preschools.

The directives from the Public Health Agency of Sweden at the time of the study included strict instructions to stay at home if any symptoms

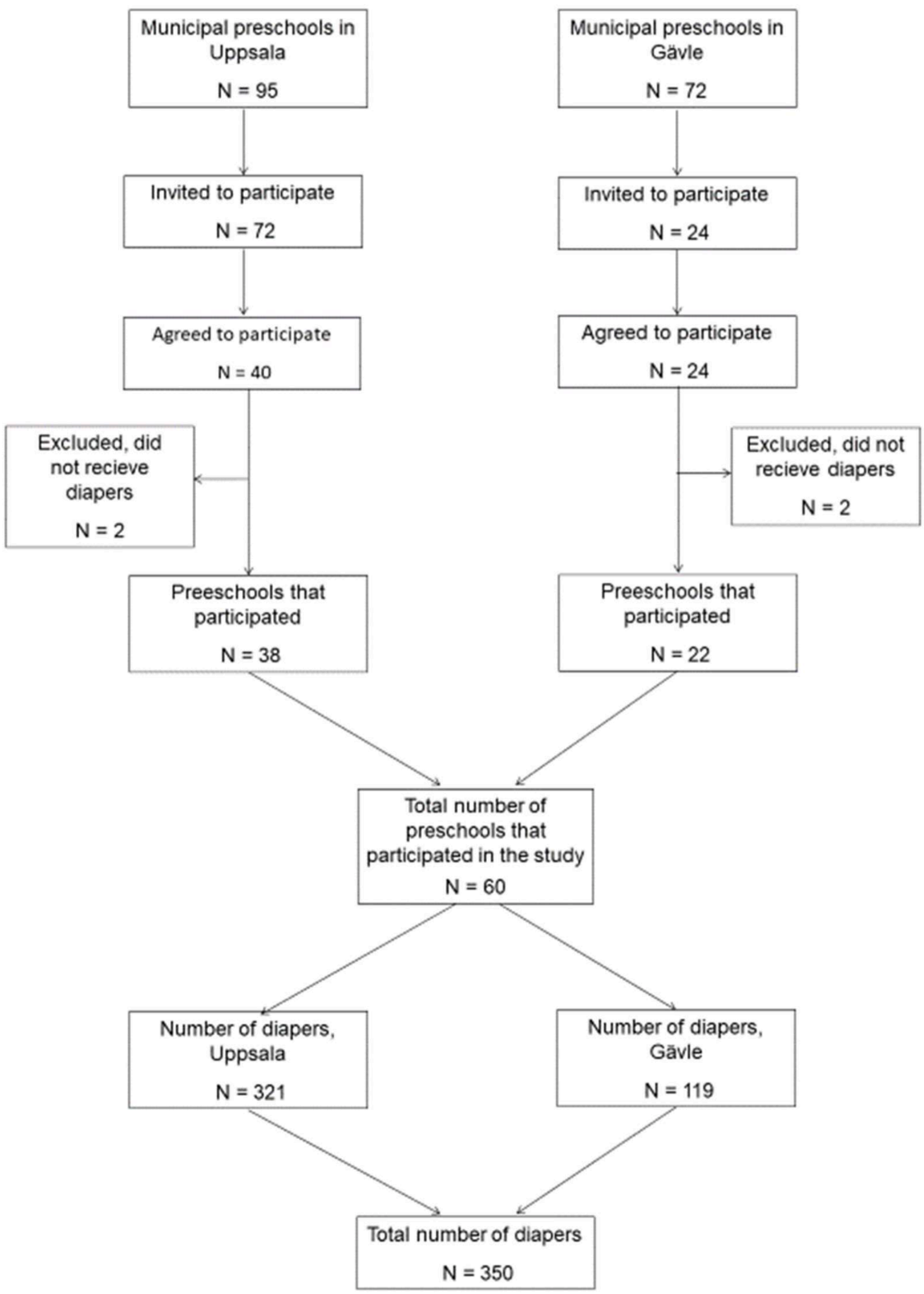

Fig. 1. Invited and participating preschools and collected diapers in Uppsala and Gävle.

of infection were present. Children with symptoms were not allowed to attend preschool. If a child developed symptoms while there, the parents were immediately notified and the child fetched. The preschool age group was not covered by the national SARS-CoV-2-screening strategy nor the COVID-19 vaccination program, and testing were performed based on medical needs alone.

2.2. Study design

Of the 95 municipal preschools in Uppsala and the 72 in Gävle, those mainly located in the central parts of each city were invited to participate in the study (Uppsala n=72 and Gävle n=24, see Fig. 1). Through the preschool principals, the staff and the children's legal guardians were informed about the study. On a predetermined day, preschool staff collected one disposable diaper with stool per child. As children with symptoms were required to stay at home, those present on the collecting day were considered healthy. To avoid duplicates, each diaper was marked with the child's age in months. No other personal data were recorded, and the children remained anonymous to the research group throughout the study. The diapers were picked up within 24 h. Information on how many diapered children were enrolled in each preschool or present during collection was not available.

Population-based epidemiological data about SARS-CoV-2 were extracted from the laboratory information systems in the Departments of Clinical Microbiology at Uppsala University Hospital and Gävle Hospital. The departments serve the hospitals and the primary care in each region. In addition, COVID-19 data reported to the Public Health Agency of Sweden were reviewed.

2.3. PCR-detection and sequencing

A loopful of fresh stool was suspended in 500 µl phosphate-buffered saline, homogenized and spun down at 2,500 rpm for 5 min. Thereafter, 200 µl of the supernatant and equal amount of MagNA Pure 96 external lysis buffer (Roche Diagnostics, Basel, Switzerland) were mixed. The extraction was carried out by using MagNA Pure 96 DNA and viral NA small volume kit (Roche Diagnostics), using the Pathogen universal program with a starting volume of 200 µl and an elution volume of 50 µl.

Detection of SARS-CoV-2 was performed by an in-house real-time reverse transcriptase polymerase chain reaction (rRT-PCR), based on the method by Corman *et al.* (modified by the Public Health Agency of Sweden, details available upon request) [15]. The rRT-PCR was verified and routinely used for clinical samples, encompassing a variety of specimen types, including feces. A sample was regarded as positive if the sample showed a reaction curve that exceeded the fixed threshold of 0.02 normalized fluorescence units. A negative control, consisting of sterile H_2O, and a positive control, consisting of a nasopharyngeal sample positive for SARS-CoV-2 were included in each run. In order to detect potential inhibitors, an additional aliquot of each extracted sample was spiked with SARS-CoV-2. rRT-PCR inhibition was defined to be present if there was an increase in cycle threshold (Ct) value $\geq$ 3.3 in the spiked sample compared to a spiked control. In the event of test inhibition, a stepwise dilution in sterile water (1:10, 1:50, 1:100) was performed and the rRT-PCR was repeated.

To further control the amplification efficacy in stool, a PCR-negative stool sample spiked with different dilutions of a SARS-CoV-2 positive nasopharyngeal patient sample was extracted and analyzed by rRT-PCR in triplicate aliquots. In comparison, the same dilution of SARS-CoV-2 nasopharyngeal patient sample with the absence of the stool sample was extracted and analyzed. In order to investigate how storage of the samples affected the outcome, each aliquot was analyzed after storage in refrigerator for 24 h, 48 h and five days.

Ten percent of the COVID-19 positive clinical samples from the Uppsala region were sequenced routinely by the laboratory, using GridION (Oxford Nanopore Technologies, Oxford, Great Britain). At the time of the study, there were no sequencing facilities in Gävle and a selection of the positive clinical samples (at least 10%, mostly from hospitalized patients) were sent to an external laboratory for sequencing.

3. Results

3.1. Study group

Altogether, 64 out of 96 (67%) invited preschools consented to participate in the study, 40 out of 72 (56%) in Uppsala and 24 out of 24 (100%) in Gävle (see Fig. 1). Four preschools (Uppsala n=2, Gävle n=2) lacked diapers with stool on the day of collection and were therefore excluded from the study. The recruited preschools represented various city districts with different socioeconomic compositions.

From the remaining 60 preschools, a total of 350 individual diapers were obtained: 231 (66%) from Uppsala and 119 (34%) from Gävle (Fig. 1). The median age of the children was 24 months in Uppsala and 27 months in Gävle (range 12-46 and 14-72, respectively), see Fig. 2. Sixteen diapers had no age specified (Uppsala n=12, Gävle n=4).

3.2. rRT-PCR

Evaluation of amplification efficacy showed that PCR-products were detected in all dilutions of spiked stool samples (Ct-range 23.9-37.0) and this was comparable with the Ct-values in the nasopharyngeal samples with the absence of stool (Ct-range 22.6-36.7). The Ct-values for the spiked stool samples were stable during the tested time-period.

SARS-CoV-2 RNA was not detected in any of the 350 samples analyzed. Ct values between the spiked aliqoutes and spiked control was compared in order to detected potential inhibitors. In 168 (48%) samples, no inhibition was detected as the Ct value increase was less than 3.3 compared to Ct value of the spiked control and these samples were therefore analyzed undiluted. To eliminate potential inhibitory effects in the remaining samples, 168 (48%) samples had to be diluted 1:10, 13 (3.7%) were diluted 1:50, and 1 sample (0.3%) was diluted 1:100 (see Fig. 3).

3.3. Epidemiology

In 2021, COVID-19 generated 871,512 reported infections in Sweden [16]. One major wave of the infection had occurred prior to the study, in April-May 2021. There was also a minor wave in August-September. The epidemiological situation of COVID-19 in the two regions (total number of tests performed at the laboratories in Uppsala and Gävle and the proportion of positive COVID-19) during June 2021 to January 2022 is shown in Fig. 4.

At the start of the study, a total of 5,833 COVID-19 cases (56 cases/100,000 inhabitants) were reported in Sweden (weekly reports from the Public Health Agency) [17]. At the end of the study period, this figure had increased to 24,563 (237 cases/100,000 inhabitants). The percentage of positive tests also increased during the same time period, from 5.9% to 6.4%. The number of Swedish children aged 0-9 years reported to be COVID-19 positive was 84 cases/100.000 inhabitants in the beginning and 452 cases/100.000 inhabitants at the end of the study. The preschool group was likely underrepresented in the statistics because they were advised to stay home if they showed symptoms, without undergoing any testing. The most rapid increase among children during this time period was noted in the group aged 10-11 years (791 cases/100,000 inhabitants).

The course of COVID-19 in the Uppsala and Gävle regions was similar to that on the national level [17]. During the first study week a total of 223 cases (57 cases/100,000 inhabitants) were reported from the Uppsala region and 195 (68 cases/100,000 inhabitants) from the Gävle region. In calendar week 50, the corresponding figures were 1,175 cases (303 cases/100,000 inhabitants) and 638 cases (222/100,000 inhabitants), respectively, a three- to fivefold increase. The percentage of

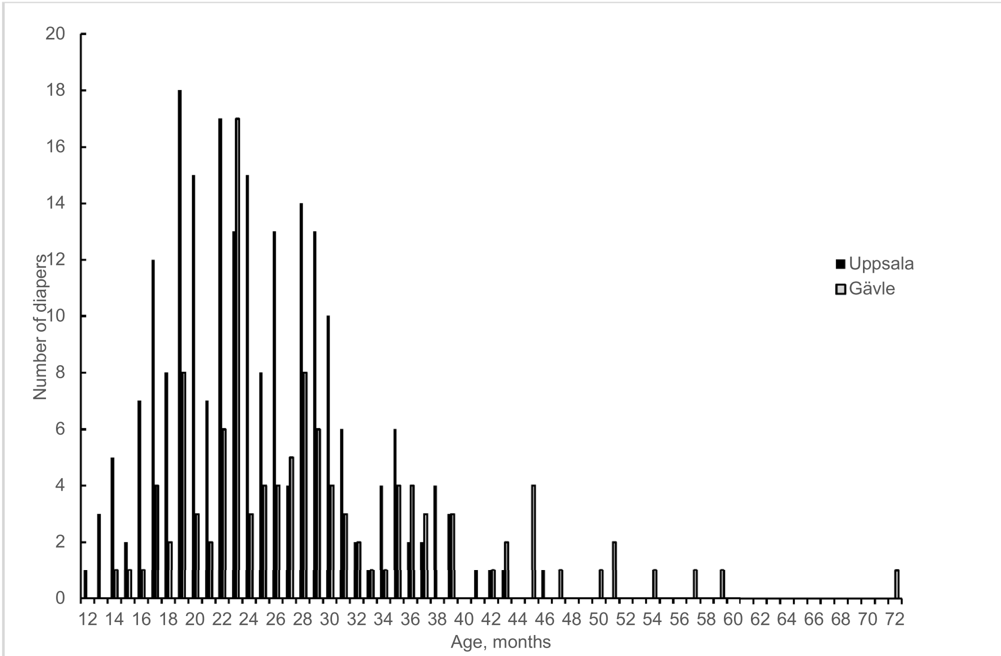

Fig. 2. Age distribution of the children included in the study.

positive tests increased during the same time period, from 6.0% to 7.1%, in the Uppsala region, whereas it remained mainly the same in the Gävle region (5.8% to 5.5%).

The total number of samples analyzed for SARS-CoV-2 in the age group 0-17 years in the Uppsala region was 8,348 during the study period. Of these, 723 samples obtained from 700 patients were positive by rRT-PCR. Amongst the youngest children (aged 0-5 years), 67 samples were collected during the same time period. Eighteen of them were rRT-PCR positive, of which 16 were sampled from children in the preschool age (1-5 years). The Ct-values of these 16 samples had a median of 21.6 (range 10.6-31.9).

During the same time period, a total of 11,834 samples were collected from individuals aged 0-17 years in the Gävle region. Of these, 813 were rRT-PCR positive, and were collected from 635 children. Of the children sampled, 1002 samples were from children aged 0-5 years, of which 40 children tested positive, all of whom were in the preschool age group (1-5 years). Ct-values for the Gävle samples were not available.

The delta variant (B.1.617.2 or 21A) predominated in Sweden throughout the study period, but the omicron variant (B.1.1.529 or 21K) emerged at the end of it and caused on average 9% of the Swedish infections at this time point (weekly reports from the Public Health Agency of Sweden) [17]. It soon became the domineering variant, and was the major finding during the fifth wave that peaked in February 2022. In Uppsala, it was identified in the beginning of December, but it did not appear among children until the middle of December. Fourteen children were infected with the omicron variant in December 2021. Eight (57%) of them had their samples collected the two last days of December. The youngest was a 2-month old baby. The remaining children were all in the age span 7-17 years (median 11 years). The Ct-value was above 20 for all these patients (median 25.5, range 22.3-29.1). In Gävle, the omicron-variant was first detected in late December. Data about this patient's age was not available.

4. Discussion

In the present study, the frequency of SARS-CoV-2 in stool samples from healthy Swedish preschool children was investigated and laboratory data were reviewed. The results showed that preschool children and their diapers were not a likely silent source of SARS-CoV-2 since the virus was not detected in the stool from any of the 350 participants. Furthermore, not even a handful of the respiratory samples from preschool children had a Ct-value below 10 and the omicron variant appeared relatively late in this age group. The probability that preschool children have played a major role in the dissemination of COVID-19, especially during the initial phase of the fifth and major wave of this virus in Sweden, is therefore low. These findings are in accord with other COVID-19 studies involving children [18–20] but in contrast to influenza, for which the epidemiologic data suggest that preschool children can serve as super-spreaders [21].

Respiratory tract samples are considered optimal specimens when diagnosing SARS-CoV-2 infections. Although SARS-CoV-2 is also shed in stool. In a previous study, the majority of children subjected to stool testing were positive for SARS-CoV-2 [12]. Furthermore, the duration of viral shedding in stool exceeded the shedding from the respiratory tract [12,22,23]. In a systematic review by Li and co-workers [23], the shedding time of SARS-CoV-2 RNA was between 28-42 days in 60% of the pediatric cases, and in 2% it lasted more than 56 days. The prolonged fecal shedding makes it therefore possible to confirm a pediatric infection for a longer time period. Additionally, recent findings have demonstrated that monitoring SARS-CoV-2-RNA in wastewater serves as an effective method to track the pandemic, and peaks in RNA-levels precedes a corresponding rise in COVID-19 hospital admissions by one to two week [24]. This was partly the rationale behind the use of diapers, but there are more advantages with diapers compared with respiratory samples: the sampling does not cause any discomfort for the participants and it does not require trained healthcare professionals.

Despite the prolonged detection of SARS-CoV-2 in stool samples, all samples were negative by rRT-PCR. PCR inhibition [25] and degradation of viral RNA [26] can be problems related to stool, but all the included controls yielded the expected results and inhibition was resolved by dilution of the affected sample. Rather, to find a case in the study population a COVID-19 frequency of at least 1:350 was necessary, a frequency that was only observed in Uppsala at the end of the study period and when all age groups were considered. The frequency of asymptomatic infections was probably even lower and about 30% [8]. That it can be difficult to detect SARS-CoV-2 during a period of low community incidence, and with prevention measures in place, was

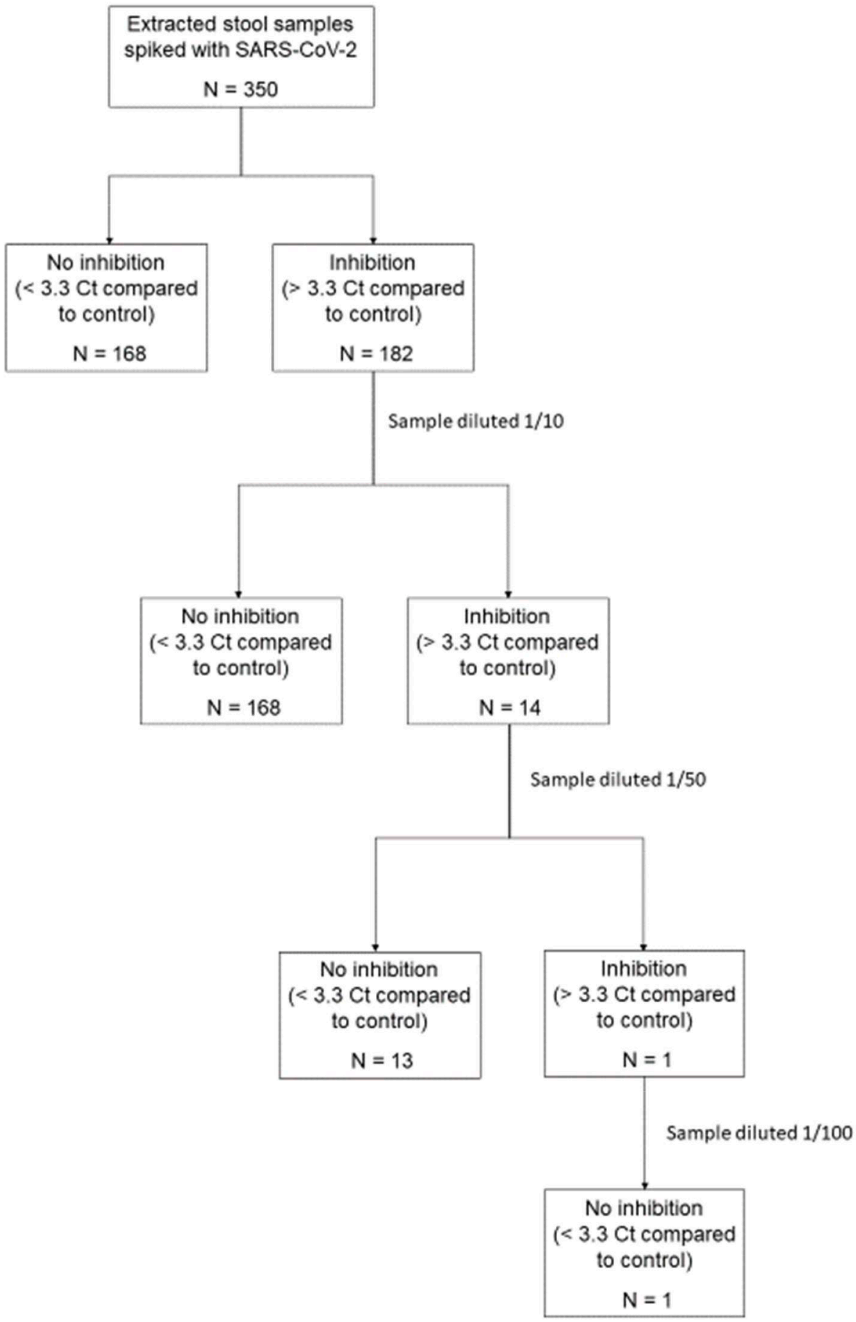

Fig. 3. Procedure for assessing and eliminating potential inhibition in the samples.

recently shown in a German day care center stud [20].

COVID-19 outbreaks in childcare facilities do, however, occur [27, 28], but a range of studies have demonstrated limited risk of facility-associated child to child transmission and outbreaks [29–32]. It is rather the staff-to-staff transmission that has an impact [19]. An increase in severe occupational injuries among Swedish preschool teachers was observed, from 2.7 cases/1,000 employed in 2018 to 3.3 cases/1,000 employed in 2021 [33]. A comparable rise could not be observed among nurses, assistant nurses, healthcare assistants, and other types of teachers. The background to the increase was due to the national recommendations of that time; to decrease the risk of dissemination of COVID-19, the outdoor activities increased considerably and more accidents than normal followed.

The preschool environment is challenging in terms of infection control, and especially in areas with low socioeconomic status [34]. Young children interact in close proximity with people, and implementing infection control measures is difficult. Despite this fact, the Swedish government decided to keep the preschools open throughout the pandemic, whilst promoting outdoor activities, good hand hygiene, attention to cleaning/ ventilation and vaccination among certain groups. Children were, in addition, only allowed to attend if they exhibited no symptoms of infection, a rather blunt recommendation that disregarded asymptomatic infections. Kim *et al.*[29] reported that facilities with asymptomatic COVID-19-cases are at increased risk of an outbreak, whereas Haag and colleagues [31] found no evidence of relevant asymptomatic spread during low or high pandemic periods. This study did not observe any asymptomatic cases at all, why this recommendation maybe was sufficient.

The present study has both strengths and limitations. One strength is that it was performed in a country with unique national

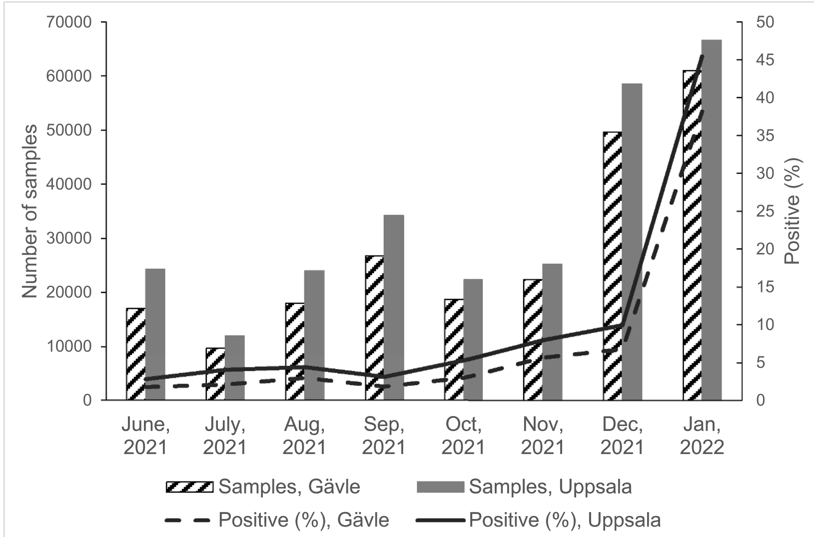

Fig. 4. Epidemiological situation for COVID-19 in Uppsala and Gävle during June 2021 to January 2022.

recommendations. Prolonged closures of preschools could therefore not interfere with the results, which would have been the case in most countries. Furthermore, it was carried out during an interesting and hard to catch phase of the COVID-19 pandemic with the emergence of one of the most contagious variants so far, it covered all socioeconomic areas in two cities with different characters, and the selection bias was reduced by using diapers. There are also some limitations, including lack of data regarding previous COVID-19 infections among the participants and whether, and to what extent, each preschool had implemented infection control measures. Additionally, there was a dropout of preschools, with stated reasons including a strained working situation, study fatigue following previous participation in similar surveys, and an outbreak of gastroenteritis.

5. Conclusion

In conclusion, SARS-CoV-2 was not detected in any of the 350 stool samples collected from healthy preschool children during a period when the delta variant still predominated but the fifth wave caused by omicron had started. This finding, together with laboratory data, suggests that this age group is not a leading propagator of this virus. Furthermore, asymptomatic children and their diapers did not constitute a silent source of SARS-CoV-2 under the given circumstances.

Ethics approval

No ethical approval was needed as the study material was considered biological waste and participants could not be identified.

CRediT authorship contribution statement

Anna Heydecke: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Susanne Sütterlin:** Writing – review & editing, Visualization, Supervision, Investigation, Formal analysis. **Åsa Melhus:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Karolina Gullsby:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Thanks to the participating pre-schools for their invaluable help.

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