KU LEUVEN

LEUVEN UNIVERSITY
VACCINOLOGY CENTRE

sciensano

INTERIM REPORT

Seroprevalence of SARS-CoV-2 antibodies in school aged children in two regions with difference in prevalence of COVID-19 disease

Validation study of saliva test for SARS-CoV-2 antibodies in children

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RESEARCHERS

Prof. dr. Corinne Vandermeulen
dr. Lise Boey
dr. Mathieu Roelants

Leuven University Vaccinologiy Centre
KU Leuven
Kapucijnenover 35, PO 7001
3000 Leuven

dr. Els Duysburgh
dr. Isabelle Desombere

Sciensano
Juliette Wytsmanstraat 14
1050 Elsene
Belgium

Dr. Joanna Merckx
Mc-Gill University
Purvis Hall
1020 Pine Ave. West
H3A 1A2 Montreal, QC,
Canada

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Dankjewel!

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LIST OF ABBREVIATIONS

CI	Confidence interval
CLB	Centrum voor Leerlingen Begeleiding
COVID19	Disease following infection with the SARS-CoV-2 virus
ELISA	Enzyme-Linked Immunosorbent Assay
SARS-CoV-2	Severe Acute Respiratory Syndrome – Corona Virus 2

SUMMARY

A new coronavirus emerged at the beginning of 2020 giving rise to a pandemic not experienced since the Spanish flu over 100 years ago. In children PCR-confirmed cases of COVID-19 might underestimate the real extent of the infection incidence in this group since less tests are done in children because of mild disease or asymptomatic infection. Seroprevalence studies indicate the number of people within a study population that have been infected in the months before the sampling and might thus give a better idea about the actual disease burden. Furthermore, it is not known whether regional differences in incidence are reflected in the seroprevalence of antibodies in children.

We investigated the seroprevalence of antibodies against SARS-CoV2 in children from the primary schools and the first three years of secondary school from two Belgian municipalities, Alken and Pelt. Alken was a hotspot during the primary wave of the COVID-19 epidemic in the spring of 2020 with a prevalence of 18.2 confirmed cases/1,000 inhabitants. The other municipality, Pelt, had a lower prevalence of confirmed SARS-CoV-2 infections with 3.3 confirmed cases per 1,000 inhabitants (prevalence beginning of July 2020). In total, 362 children were enrolled of which 90 in primary schools in Alken, 91 in primary schools in Pelt, 91 in secondary schools in the vicinity of Alken and 90 in a secondary school of Pelt.

An e-mail containing the link to the questionnaire was sent to all 362 parents of children with a serum sample and the online questionnaire was completed by 341 of these parents (94.2%) between 21 September and 19 October 2020.

The current report gives details about the seroprevalence of SARS-CoV-2 antibodies in the children from whom a blood sample was taken.

We found that the difference in incidence between the two municipalities was reflected in the seroprevalence of antibodies in the children. In Alken SARS-Co-V2 antibodies were found in 14.4% of the participants (26 out of 181), whereas in Pelt this was only 4.4% (8 out of 181). In addition, we found that in Alken there was hardly any difference between the proportion of infections in primary school children (13.3%) and adolescents in the first three years of secondary education (15.4%). Children who had had direct contact with a confirmed infected person were also found to have a four times higher risk of having antibodies compared to children whose parents reported no contact with an infected person. However, no cases of severe SARS-CoV-2 infection were reported.

We concluded that infection of SARS-CoV-2 in children might be higher than previously expected and reflects the virus circulation in the community but that the disease is mostly mild in this group.

1. INTRODUCTION

At the end of 2019 the first cases of a severe respiratory illness due to a new coronavirus, SARS-CoV-2, were reported in Wuhan, China, which has since then grown into a pandemic due to global dissemination of the virus with more than 55 million persons infected and more than 1.27 million confirmed deaths as a result of COVID-19 disease (data up to 11-nov-2020 – https://coronavirus.jhu.edu/map.html).

Despite global fast-tracked research, uncertainty remains over the epidemiological and serological characteristics of the novel SARS-CoV-2 pandemic. Our knowledge of transmission dynamics in the pediatric population is even more limited (Lee Oct 2020). The real extent of the epidemic in the general adult and pediatric population is unknown. Early investigation only recorded data of patients with severe disease for whom a PCR test was performed. Available PCR positive data can thus only give us a very selected view on the complete epidemic (Sciensano data). Mild or asymptomatic infections that did not require medical attention were hence underdiagnosed (Oran, June 2020). Among the asymptomatic cases there is a high proportion of children as they are more often asymptomatic compared to older individuals (Choi, 2020). In Belgian studies up to September 2020 estimated seroprevalence between 5 and 8% using residual blood samples, samples from healthy blood donors and samples obtained from health care workers (HCW) (Sciensano). However, major regional differences exist even in a small country as Belgium. Sero-prevalence studies have in particular been very sparse and included only a limited number of children in Belgium and internationally (Arora, 2020; https://www.covid19immunitytaskforce.ca/research/global-serological-knowledge-hub/). For this reason, it is unclear if the regional differences are fully reflected in the pediatric population.

The virus' ability to spread in the human population and individual associated risk factors equally remain unclear. The role of subclinical infections in human-to-human transmission is not well understood. Data support pre-symptomatic and asymptomatic transmission (Nishiura, 2020). Children can get infected by SARS-CoV-2, however, systematic review of publications shows they have half of the odds to be infected compared to adults. (Viner, 2020) Equally in household studies they showed a secondary attack rate half of that of adults (Madewell 2020). However, more recent data show larger secondary attack rate in children and with children as the infector in the household setting, even though the number of children remained small (Grijalva 2020). Since most of the available data originate from times in which children were at home during the country wide lock downs, it remains unclear whether apparent low numbers of infections in children are due to lesser exposure or to an inherent decreased susceptibility to infection. In an early report, most children were apparently infected within their households. Their potential to transmit the virus had been assumed based on limited available data about the current COVID-19 pandemic and (previous) influenza epidemics during which children are known to be major community spreaders.

Sciensano reported that 9,806 or 2.2% of cases occurred in <10y and 48,997 or 11.0% of cases in children 10-19y with four deaths were reported in the age group 0 to 24 years. (Sciensano report of 3 Nov 2020) Similarly, other Western countries have recorded lower number of cases in children compared with other age groups. The lower reported number of cases in children can be partially explained by the lower testing rates for the diagnosis of acute infection in the pediatric population. This leads to selection bias in the effect measures calculated based on tested individuals only.

Different study designs can provide information on the transmission dynamics in children. One of these is the study of the seroprevalence and incidence in the pediatric population. Large population wide serosurveillance studies have been performed in Switzerland and Spain. (Stringhini, 2020; Pollan, 2020) The proportion of children in these studies is, however, small and prone to selection bias. To obtain a measure for the prevalence of past SARS-CoV-2 infection in school-aged children, it is necessary to sample children age-appropriately. Although serology studies will not be able to provide a complete and direct answer regarding transmission dynamics, they provide cumulative data on past infection which will help our understanding of SARS-CoV-2 in children and allow to follow-up the direct effects in this population.

In order to facilitate large population studies in school aged children an easy-to-use, non-invasive method to measure antibody titers against SARS-CoV-2 should be developed, especially if multiple samples in the same population will be taken over time. Determination of antibodies in saliva has been used often, for instance for measles and mumps (Braeye 2013) and dengue. Recent, data on the accuracy and agreement of the detection of salivary antibodies compared to the sero-prevalence and antibody titers in serum in an adult population have been published (Isho, 2020; Pisanic B 2020) and find a good agreement of IgG titers and detection of past infection in salivary specimens. The saliva test was as well already validated in adults in the Laboratory of Sciensano, using the available IgG commercial immunoassays (Wantai and Euroimmune) and proved to have a good correlation with the serum test.(unpublished data) However, prior to setting up large population studies in children, the saliva method used to determine antibodies against SARS-CoV-2 should be validated in this pediatric population because antibody levels may present differently in children and sample taking might be more difficult because children produce less saliva. This requires comparing the index saliva test against the reference standard, which is antibody determination in serum and its ability to correctly detect prior infection of SARS-COV-2 to determine sero-prevalence and incidence in school aged children on a population level.

2. OBJECTIVES OF THE STUDY

This study was set-up with a dual purpose. The main purpose was to validate saliva as a valid sample for antibody detection which will be used in a larger study on the seroprevalence and incidence of SARS-CoV-2 in school-aged children in Belgium. In addition, we wanted to compare the seroprevalence of antibody titers against SARS-CoV-2 in children in two regions which largely differ in the prevalence of confirmed SARS-CoV-2 infection in the early phase of the pandemic. Findings will be helpful for relevant experts to guide their advice regarding measures to be taken in children and youngsters during resurgence of the SARS-CoV-2 virus.

Objectives

Primary objective:

- Validate the saliva sample (index test) for antibody detection (IgG) against SARS-CoV-2 using an ELISA test compared to the reference standard of antibody test based on venous blood sampling and serum testing (agreement).

Secondary objectives:

- Assessment of accuracy of saliva testing for the detection of antibodies against SARS-CoV-2 compared to the reference standard of serum-based ELISA testing.
- To determine the seroprevalence of antibodies against SARS-CoV-2 in school-aged children (primary school and first grade of secondary school) in a region with a high prevalence and a region with a low prevalence of confirmed SARS-CoV-2 infection in Limburg.
- To compare the prevalence of antibodies against SARS-CoV-2 in school aged children between these two municipalities.
- Determine the prevalence of children living in a household with (past) confirmed or probable COVID-19 cases based on a questionnaire.

3. METHODS

This is a cross-sectional study in which children were prospectively recruited in two municipalities in Limburg with different epidemiological background of SARS-CoV-2 infections.
The study measured past contact with the SARS-CoV-2 virus in school aged children (primary school and first grade of secondary schools) by measuring antibodies in both serum and saliva.

3.1 Population and recruitment

The study covered two municipalities in Limburg including one, Alken, which was a hotspot during the primary wave of the COVID-19 epidemic in the spring of 2020. Alken had a prevalence of 18.2 confirmed cases/1,000 inhabitants. The other municipality, Pelt, had a lower prevalence of confirmed SARS-CoV-2 infections with 3.3 confirmed cases per 1,000 inhabitants (prevalence beginning of July 2020).
Per municipality 180 children were enrolled, of which 90 primary school children and 90 young teenagers from the first to third year of secondary school, totaling 360 school-aged children.
Children and youngsters from Alken and Pelt were recruited at school and selected independent of prior known exposure to SARS-CoV-2 cases and/or infection. Given study participation depended on parental and child consent, there was partial self-selection and potential exposure can affect willingness to participate in the study.

In- en exclusion criteria were the following:
Inclusion criteria

- Being a child aged between 6 and 12 year or 12 and 15 years
- Domiciled in either Alken or Pelt
- Signed informed consent form of parents and informed assent of child

Exclusion criteria

- Children with contra-indication for venous blood sampling or saliva sampling (e.g. bleeding disorder, low platelet count, severe immune suppression)

The schools in Alken and Pelt were contacted by the youth doctors of the school health services (CLB) of Limburg. Since there is no secondary school in Alken, the secondary schools in the neighborhood (Hasselt, Stevoort and Herk-de-Stad), where adolescents from Alken go to, were contacted by the youth doctors. Children and their parents were invited to participate in this study through a letter that was distributed in the participating schools.
In total 90 children per age group and per municipality were allowed to participate. Parents returned a completed participation form to the schools, which was forwarded to the investigators.
Since more children and their parents than this a priori calculated sample size wanted to participate, children were randomly chosen from the entire group. Nevertheless, care was taken to select as many boys as girls, evenly spread over the different age groups and to choose only one sibling per family.

3.2 Determination of the sample size

Test validation
The sample size is determined by the precision of the sensitivity and specificity of the test (validation) and informed by priors defined by the available PCR test-data and assumptions on the background exposure and expected prevalence in the two regions. One high background exposure (Alken) region and one low background exposure (Pelt) were selected, accounting for:

- Confirmed cases: Alken, 18.2 /1,000 inhabitants; Pelt, 3.3 /1,000 inhabitants (cases from Sciensano, 14/06/2020, size of the population from Statbel 1/1/2020); Alken is at the top (highest

prevalence discounting Herstappe which had 3 cases in a total population of 79); Pelt is near the first quartile (p25) (the median number of cases is 4.4/1,000) (data beginning of July 2020)
- Assumed number of seropositives: approximately 15% in Alken, 4% in Pelt (cfr. data University of Antwerp, 6-7%). The seroprevalence in children is assumed to be half this number: 8 and 2%, or overall ~ 5% in the present study

For the validation this would, given the above priors and assumptions, result in approximately 5 seropositive cases per 100 tests, or 20 cases and 380 seronegatives out of 400 children tested;
Assuming a perfect test (sens = spec = 100%):

- N = 20 children with antibodies present tested gives a lower boundary for the sensitivity of 80% (73-100% when the sensitivity is 95% and 69-98% when the sensitivity is 90%);
- N = 380 negatives tested gives a lower boundary for the specificity of 99% (92-97% when the specificity is 95% and 86-93% when the specificity is 90%);

While this may appear unbalanced with respect to the lower precision achieved for the sensitivity, it is important to realize that the saliva test will be validated for serosurveys (with a low prevalence), and not as a diagnostic tool in individuals with a higher pre-test probability. A survey of antibodies in a population with a low prevalence (say, 5%) is much more affected by a low specificity (e.g. the prevalence could double when the specificity is 'only' 95%) then it is by a low sensitivity (the prevalence will decrease with max. 1% when the sensitivity is only 80%).
Because we aimed at recruiting equal numbers of primary and secondary (1 – 3rd year) schoolchildren in both regions, and assumed that about half of primary schoolchildren could be recruited, the total sample size was reduced to 360: 90 primary schoolchildren (a typical primary school has about 6 x 20 = 180 children), and 90 secondary school children in each region.
With the present sample size, we can statistically prove a difference in proportion of cases of 2% in Pelt vs. a 8% in Alken with a power of 80% and significance level of 0.05. In addition, we can demonstrate a difference of 4% in primary schoolchildren vs. 12% in secondary schoolchildren, e.g. in case the latter group has a prevalence that is more comparable to that in adults.

3.3 Questionnaire

An online questionnaire was drafted in July 2020 and the online version of the questionnaire was entered in Limesurvey of Sciensano (LimeSurvey Project, Hamburg, Germany, URL http://www.limesurvey.org), a tool for online questionnaires beginning of September. Once the questionnaire was complete, it was piloted with parents with children from the same age group for readability as well as time to complete the questionnaire.
The questionnaire consisted of five parts and collected data on socio-demographic determinants and data on the family and housing conditions, health of the child, symptoms of COVID-19 disease (based on the case definition of Sciensano dd13-jul-2020), risk to acquire a SARS-CoV-2 infection.
The questions for socio-demographic data were based on the questionnaires used in the Flemish vaccination coverage studies. Questions related to SARS-CoV2 and COVID-19 disease were based on the current state of knowledge and equally used in household studies.
Every evening after a child was sampled, Parent One with a valid e-mail address, most of the times coinciding with the e-mail address of the mother, received an e-mail with a personal link to complete the questionnaire. Reminders to complete the questionnaire were sent after one week. For the remainder of the parents who did not respond, the mail with the link was sent to the other parent when an e-mail address was available.
An additional questionnaire to the parents with questions related to the profession of parents as well as travelling of household members to regions or countries with high prevalence of transmission at the time of travelling, was sent on 26-oct-2020.
Only questionnaires for which all questions were answered were considered for analysis. The questionnaires were completed prior to reporting of the individual test results to the parents and thus blinded for the outcome of prior infection.

3.4 Collection of biological samples

In all children both a regular blood sample was obtained by venipuncture as well as a saliva sample by using an Oracol device (Malvern Medical Developments, UK).
In the Oracol-based sampling method a cylindrical plastic sponge mounted on a short wooden stick was placed between the gums and the cheek and the child was asked to move or rotate the sponge for 1.5 to 2 minutes on the gum of the teeth (instruction document for nurses).
Venipuncture was carried out by puncturing the skin with a needle at an appropriate site (most often the elbow pit) where easy access to a vein is found. Per child one dry serum tube of 10 ml was taken.

Each sample was labelled with a specific code per child.
Blood and saliva were stored at room temperature until pick-up by the driver for transport to the laboratories of Sciensano. Samples were transported to the laboratory on a daily basis.
At the laboratory, blood samples were centrifuged for 10 to 15 minutes at 1300G after which serum was aliquoted into tubes of 1.5 to 2 ml and stored at -20°C.
Both the saliva index test and the blood reference test were run at the Sciensano laboratory of Immune response. Laboratory test processors did not have clinical information of the child and the index test and reference test were ran independently and blinded to the pairwise test result.

3.6 Laboratory testing

Diagnostic testing and specimen selection

The current assays that are routinely used at the laboratory of Sciensano are the Euroimmun (Euroimmun, Medizinische Labordiagnostika, Lübeck, Germany; Cat # EI 2668-9601 G; CE marked March 2020) enzyme-linked immunosorbent assay (ELISA) and the Wantai SARS-CoV-2 Ab ELISA (cat n° WS-1096; Beijing Wantai Biological Pharmacy Enterprice Co. Ltd., China). The Euroimmun ELISA measures human IgG antibodies targeting the S1 domain including the receptor binding domain (RBD) of the structural protein of SARS-CoV-2. The Wantai-ELISA measures antibodies directed to RBD and detects anti-RBD IgG, IgA and IgM concomitantly. Both assays are medium through-put ELISA requiring in-laboratory testing. The result is read using a microplate reader. As per the manufacturer a ratio is calculated between the optical density (OD) of the patient sample compared to the OD of the calibrator. For the Euroimmun ELISA, a ratio of <0.8 is interpreted as negative, between 0.8-1,1 borderline and >1,1: positive, for serum samples. Serum, EDTA plasma and capillary blood (dried blood spot) specimens have been validated by the manufacturer. Accuracy data provided by the manufacturer give a sensitivity of 94.4% (no confidence interval given, n=72, adult samples) and specificity of 100% (in 74 children tested) (https://www.coronavirus-diagnostics.com/antibody-detection-tests-for-covid-19.html) using known negative and positive serum samples. Clinical laboratory validation on adult patients showed a specificity of 100%, with 50 out of 50 samples negative (Charlton et al, 2020) Sensitivity using known positive samples showed an overall all point sensitivity of 63% (95% CI 46-77) and of 88% (95%CI 46-100) in patients more than 21 days post PCR confirmed SARS-CoV-2 infection. Overall, combining literature data (Van Elslande et al, 2020) and in house validations, a specificity of 98,6% (494/501; 95%CI 97,1-99,3) and a sensitivity of 95,9% (95%CI 88,8-98.9) at ≥ 15 days post onset of symptoms was observed for the Euroimmun IgG Elisa taken a ratio of 1,1 as cut-off (accuracy 98,3% (565/575).
For the Wantai ELISA, the manufacturer's recommendations for seropositivity are ratio ≥1.0 for anti-RBD Ig seropositivity. Using this cut-off and based on *in house* and reported validations (Lassaunière et al, 20020; Geurts-van Kessel et al, 2020; Bastos ML et al, 2020; Elslande JV et al, 2020), the estimated specificity and sensitivity, measured >14 days post onset of symptoms are 99.6% (772/775; 95%CI 98.9-99.9) and 100% (155/155; 95%CI 97.6-100), respectively.
Laboratory technicians were blinded for all information from the questionnaire completed by the parents.

3.7 Ethical committee

On 22 July 2020, the protocol of the study and related documents were submitted to the Ethics Committee Research of UZ/KU Leuven (S64415). A positive advice to conduct the study was received on 11-sep-2020.
On 19-Oct-2020 the additional questionnaire was also submitted for ethical review and positive advice to use the questionnaire was received on 21-oct-2020.

3.8 Data analysis

Data were transferred from Limesurvey to R for further analysis. Answers to open ("other", …) questions were assigned to the predefined answer categories where possible, or otherwise grouped as 'other'. Where possible, answer categories were aggregated to make interpretation of the data more straightforward. Inconsistent or erroneous administrative data were corrected.

The origin of the child was classified as *Belgian* when both parents and at least two grandparents were born in Belgium; as *Dutch* when a parent or at least two grandparents were born in the Netherlands, as *European* when a parent or two or more grandparents were born in a European country, and as *non-European* when a parent of two or more grandparents were born outside Europe. One child with an Australian origin was classified as "European"; other non-European origins were Asian, African, or South-American.

3.9 Statistical analysis of the data

Descriptive statistics of sample characteristics are presented as frequencies and percentages for categorical variables, or as means and standard deviations for continuous variables. These categorical variables were compared between regions or groups using a chi-square test or fisher exact test as appropriate, and continuous variables with a t-test. No correction for clustering by class or school was applied for the analysis of sample characteristics.
The analysis of serologic outcomes (SARS-CoV-2 antibodies) was adjusted for clustering of children in school and classes given the highly infectious nature of the disease under study. Standard errors and confidence intervals of prevalence estimates were estimated with generalized estimating equations (GEE) with a binomial distribution and exchangeable correlation structure (compound symmetry). Clusters were defined by the class of the current schoolyear. An identity link function was used for the prevalence and risk difference (RD), and a log link function for relative risks (RR). Log transformed estimates and confidence intervals were backtransformed (exponentiated) for reporting. For the analysis of risk factors models were additionally adjusted for region and school levels (primary, secondary) when this was deemed relevant (when the prevalence of risk factors differed according to these variables). The data were analyzed with the statistical software package R (version 4.0.0, R Foundation for Statistical Computing, Vienna, Austria, 2020). GEE's were estimated with the geepack package (Halekoh U, Højsgaard S, Yan J (2006) The R Package geepack for Generalized Estimating Equations. Journal of Statistical Software, 15(2):1-11) package in R).

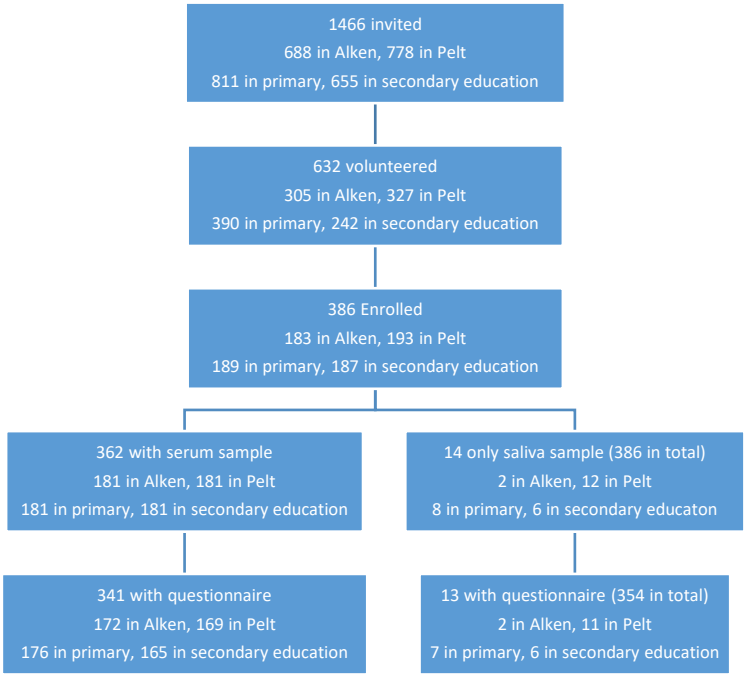
4. RESULTS

4.1 Description of the population

4.1.1 *Composition of the sample*

Children and adolescents and their parents living in two municipalities, Alken en Pelt, were targeted to participate in this study. Therefore, two primary schools out of nine and one secondary school out of two in Pelt were invited to participate in this study. In Alken there is no secondary school and therefore secondary schools in the vicinity of Alken were adolescents from the village go to school, were invited to participate along with three primary schools out of six located in Alken. Selection was based on willingness to participate asked by the contact person of the school health services of Limburg.

In total, 1466 children and their parents were invited to participate in this study, 632 (43.1%) volunteered, 376 (25.6%) were enrolled, and from 362 children (24.7%) a blood sample was taken of which 90 in primary schools in Alken, 91 in primary schools in Pelt, 91 in secondary schools in the vicinity of Alken and 90 in the secondary school of Pelt (figure 1).



An e-mail containing the link to the questionnaire was sent to all 362 parents of children with a serum sample and the online questionnaire was completed by 341 of these parents (94.2%) between 21 September and 19 October 2020.
The additional questionnaire was completed by 248 parents (68.5% of children with blood sample).

In total, there were 27 to 32 (range) children per grade in primary school (13 to 16 boys; 12 to 18 girls); 80 (43 boys) in first year of secondary school; 76 (32 boys) in 2nd year of secondary school and 25 (11 boys) in 3rd year of secondary school. The third year of secondary school was only sampled in Alken.(Table 1)

Table 1: Number of participating pupils per class and school:

		Primary education						Secondary (1st – 3rd year)			
	Grade:	1	2	3	4	5	6	1st sec	2nd sec	3rd sec	Total
Alken	PRIM1	5	2	2	7	8	1				25
	PRIM2		7		2	2	14				25
	PRIM3	9	8	10	6	7					40
	SEC1							9	7		16
	SEC2							6	4	3	13
	SEC3							2*	4		6
	SEC4							18	16	22	56
	Total	14	17	12	15	17	15	35	31	25	181
Pelt	PRIM1	10	11	10	10	8	8				57
	PRIM2	5	4	5	6	7	7				34
	SEC1							45	45		90
	total	15	15	15	16	15	15	45	45		181
total		29	32	27	31	32	30	80	76	25	362

* One girl with unknown class was assigned to the first year of secondary school (most likely grade for her age)

4.1.2 Demographic data on class and schools

The sample includes 175 (48.3%) boys and 187 (51.7%) girls; 86 (47.5%) boys and 95 (52.5%) girls in Alken, 89 (49.2%) boys and 92 (50.8%) girls in Pelt. The mean age at the time of sampling was 10.9 (Standard Deviation: 2.5) years (range 5.7 – 14.7 years), and did not significantly differ according to sex (p = 0.7) or region (p = 0.4).

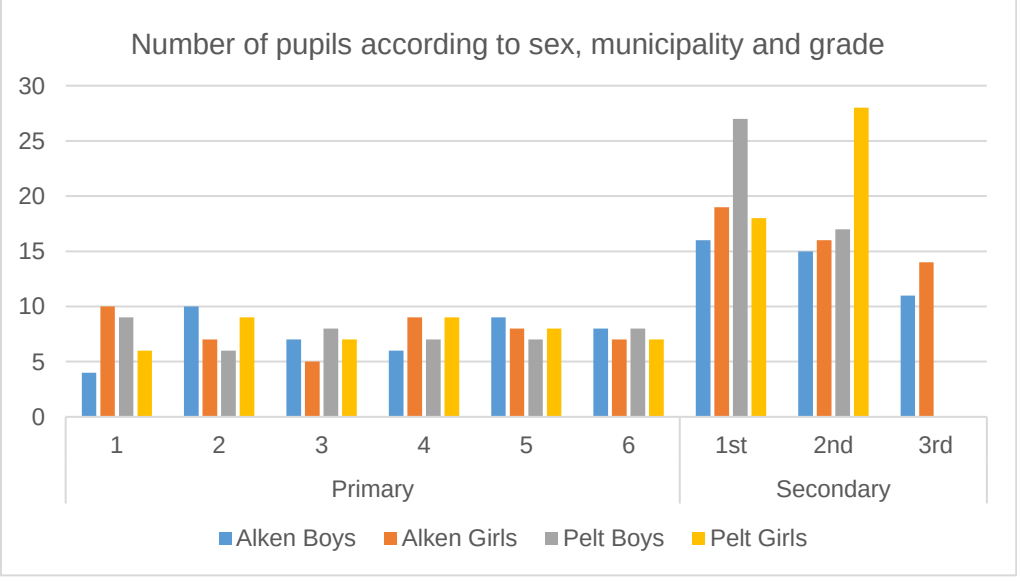


Figure 2: number of pupils according to sex, municipality and grade

In total, 25 pupils (6.9%) were delayed in their school education as per their age, 8 in primary (4.4% of primary) and 17 in secondary school (9.4% of secondary) and 5 (1.4%) were advanced in their school education with a maximum of 1 year. There were no significant differences according to region or sex.

4.1.3 Demographic data on the child, their family and surroundings

In total, parents of 341 (94.2%) children completed the initial questionnaire: 172 (95.0%) in Alken, 169 (93.4%) in Pelt.

Alken and Pelt are both semi-rural regions with population of 11500 inhabitants in Alken and 32500 inhabitants in Pelt. The included children in both regions are mostly of Belgian origin, 91.3% and 84% for Alken and Pelt respectively. The larger proportion of children with a non-Belgian background in Pelt are mostly of Dutch origin (12.4%) because of the close geographical proximity and shared language (table 2).

While most of the families are structured as nuclear families, 10% of the families are single parent families and 12.2% to 14.8% (Alken and Pelt, respectively) of the children live at more than one place. Children share a bedroom in 11.7% of the total families with youngsters or children less than 15 years of age (except in 2 families where an adult shares the room with the child) (table 2 and 3).

A larger proportion of mothers from participating children in Alken has a Bachelor degree or higher, compared to Pelt. For the absolute numbers and comparisons between regions we refer to Table 2.

Table 2: Summary of sample characteristics (questionnaire)

	Alken (n = 172)	Pelt (n = 169)	Total (n = 341)
Family context			
Nuclear family	148 (86.0%)	135 (79.9%)	283 (83.0%)
Blended family	6 (3.5%)	12 (7.1%)	18 (5.3%)
Single parent	16 (9.3%)	18 (10.7%)	34 (10.0%)
Other [a]	2 (1.2%)	4 (2.4%)	6 (1.8%)
Child lives also elsewhere [b]	21 (12.2%)	25 (14.8%)	46 (13.5%)
Education mother			
Lower, none or unknown	2 (1.2%)	4 (2.4%)	6 (1.8%)
Secondary	25 (14.5%)	54 (32.0%)	79 (23.2%)
Bachelor degree	100 (58.1%)	83 (49.1%)	183 (53.7%)
Master degree	45 (26.2%)	28 (16.6%)	73 (21.4%)
Education father			
Lower, none or unknown	0	9 (5.4%)	9 (2.7%)
Secondary	62 (36.0%)	68 (40.2%)	130 (38.1%)
Bachelor degree	66 (38.4%)	63 (37.3%)	129 (37.8%)
Master degree	44 (25.6%)	29 (17.2%)	73 (21.4%)
Origin			
Belgian	157 (91.3%)	142 (84.0%)	299 (87.7%)
Dutch	4 (2.3%)	21 (12.4%)	36 (7.3%)
Other European	2 (1.2%)	2 (1.2%)	4 (1.2%)
Non-European	9 (5.2%)	4 (2.4%)	13 (3.8%)
Total disposable income (month)			
Low (< 1500 euro)	2 (1.2%)	2 (1.2%)	4 (1.2%)
Moderate (1500 – 3000)	14 (8.1%)	20 (11.8%)	34 (10.0%)
Higher (> 3000 euro)	126 (73.3%)	107 (63.3%)	233 (68.3%)
Don't know or want to tell	30 (17.4%)	40 (23.7%)	70 (20.5%)

[a] Family context other = Co-parenting (n = 4) or foster family (n = 2)
[b] Other parent (n = 43) or grandparents (n = 3)

Most parents (339/341, 99.4%) mention Dutch as the main language spoken at home, the other two being both Dutch and Persian, or Urdu (both in Alken).

Table 3: Personal and family risk factors

	Alken (n = 172)	Pelt (n = 169)	Total (n = 341)
Child has a medical condition	10 (5.8%)	10 (5.9%)	20 (5.8%)
At-risk family members°	22 (12.8%)	26 (15.4%)	48 (14.1%)
Persons 65 or older in the household	7 (4.1%)	4 (2.4%)	11 (3.3%)
Shared bedroom*	16 (9.3%)	24 (14.2%)	40 (11.7%)
Public transport use[†]			
Regularly (> 3/week)	38 (22.1%)	8 (4.7%)	46 (13.5%)
Often 1 – 3 /week	8 (4.7%)	2 (1.2%)	10 (2.9%)
Occasionally (< 1/week)	4 (2.3%)	3 (1.8%)	7 (2.1%)
Never	122 (70.9%	156 (92.3%)	278 (81.5%)
Participated in summer camp			
Overnight camp	94 (54.7%)	88 (52.1%)	182 (53.4%)
Day camp	28 (16.3%)	34 (20.1%)	62 (18.2%)
No	50 (29.1%)	47 (27.8%)	97 (28.4%)
Extracurricular activities	153 (89.0%)	148 (87.6%)	301 (88.3%)

° at risk family members was defined in the questionnaire as a family member with an increased risk of severe COVID-19 disease 'high blood pressure, diabetes, lung disease, cardiac disease, kidney disease, immunosuppression or immune disorder

* in each region there are 2 children who share the room with a person of 15 years or older; All the others share their bedroom with another child < 15 years.

[†] Significantly different between Alken and Pelt (chi square test p<0.001)

Origin is defined by the country of birth of the parents and grandparents. If at least of these family members was born outside of Belgium, the origin of the child will be associated with this country. This classification is similar to what is done in the Flemish vaccination coverage studies.

All 341 participating children with a completed questionnaire, were reported by the parents to be in good health; 20 (5.8%) children were reported with a **chronic disease or condition**: two have a cardiovascular disease (tetralogy of Fallot; VSD), two a kidney disease, 12 asthma or allergies, 4 have an eye disease, 1 a neurological disorder (epilepsy). For only one child two conditions were reported. In this study population, 14.1% (n=48) of the children lives with a family member with conditions defined as risk factors for more severe COVID-19 disease.

Public transport use is the only characteristic that differs significantly by region. While rare (< 10%) in Pelt, almost 30% of schoolchildren in Alken make use of public transport (more than one in four at least weekly) ($p < 0.001$). Public transport is almost exclusively used by secondary schoolchildren (n = 61/168, 37.0%), versus two out of 176 primary schoolchildren (one from each region).

Extracurricular activities were followed by 48.5% (n = 82) children for more than 3h/week, 36.7% (n=62) had 1 – 3 h/week of extracurricular activities and 2.4% (n=4) < 1h/week. The distribution of the frequency is highly comparable in both regions. Extracurricular activities are a customary practice in both primary (155/176, 88.5%) and secondary (146/165, 88.1%) school. Summer camps are attended more often by primary schoolchildren (137/176, 77.8%) compared to secondary schoolchildren (107/165, 64.8%), but overnight camps are more frequent in secondary school (98/165, 59.4%) than in primary school (53/176, 30.1%), and day camps are more frequent in primary school (53/176, 47.7%) than in secondary school (9/165, 5.5%).

4.2 Laboratory analyses of serum antibodies against SARS-CoV-2

4.2.1 Seropositivity

In total 37/362 children had a positive serum antibody test with either the WANTAI SARS-CoV-2 Ab ELISA (total antibody) (n = 34; 9.4%) or the EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) (n = 35; 9.7%). Of these, 32 tested positive with both tests, 2 only with the WANTAI total antibody assay, and 3 only with the EUROIMMUN IgG assay. A comparison of the serum tests or with the oral sample is outside the scope of the present analysis.

All analyses of seroprevalence is based on the results of the WANTAI SARS-CoV-2 Ab ELISA (RBD antibody). These children are further referred as either *'positive'* or *'seropositive'*, and all others as *'negative'*. The WANTAI ELISA test was chosen as the most specific based on literature and experience within the immunology laboratory at Sciensano. The WANTAI ELISA-test measures antibodies aimed at neutralizing the SARS-CoV-2 virus at the Receptor-Binding Domain (RBD).

Overall, the seropositivity in children of primary and secondary children in the entire study was 9.4%. Table 4 gives the seropositivity of the entire group as well as per age group and municipality.

Table 4: Number of positive tests/total tested; prevalence, robust standard errors and 95% confidence interval by region and level

	Primary school	**Secondary school**	**Total**
Alken	12/90	14/91	26/181
	13.3 %	**15.4 %**	**14.4 %**
	(5.2)	(3.5)	(3.1)
	(3.2 – 23.5%)	(8.6 – 22.2%)	(8.2 – 20.5%)
Pelt	0/91	8/90	8/181
	0 %	**8.9 %**	**4.4 %**
	(-)	(3.4)	(1.9)
	(0 – 4.1%)*	(2.1 – 15.7%)	(0.7 – 8.1%)
Both municipalities	12/181	22/181	34/362
	6.6 %	**12.2 %**	**9.4 %**
	(2.8)	(2.5)	(1.9)
	(1.2 – 12.1%)	(7.2 – 17.1%)	(5.7 – 13.1%)

* Clopper-Pearson exact binomial confidence interval

Relative Risk, Risk Difference

The risk difference (RD) (secondary vs primary) was 0.01 (-0.11 – 0.13) in Alken, and 0.09 (0.04 – 0.14) in pelt; The risk difference in Alken vs Pelt was 0.14 (0.06 – 0.22) in primary school and 0.06 (0.03 – 0.16) in secondary school. The risk differences as estimated jointly for both regions and school levels is shown in table 5

Table 5: Risk difference and relative risk in Alken vs Pelt and secondary vs primary education (jointly estimated)

	Risk difference	Relative risk
Alken vs Pelt	0.11 (0.038) (0.04 – 0.18)	3.2 (1.3 – 7.9)
Secondary vs primary	0.07 (0.028) (0.01 – 0.12)	1.6 (0.7 – 3.8)

The risk was higher in Alken, and also in secondary school; both these conclusions are at least in part attributable to the zero prevalence in primary schoolchildren in Pelt.

Figure 3 and tables 6 show a further breakdown by grade and school.

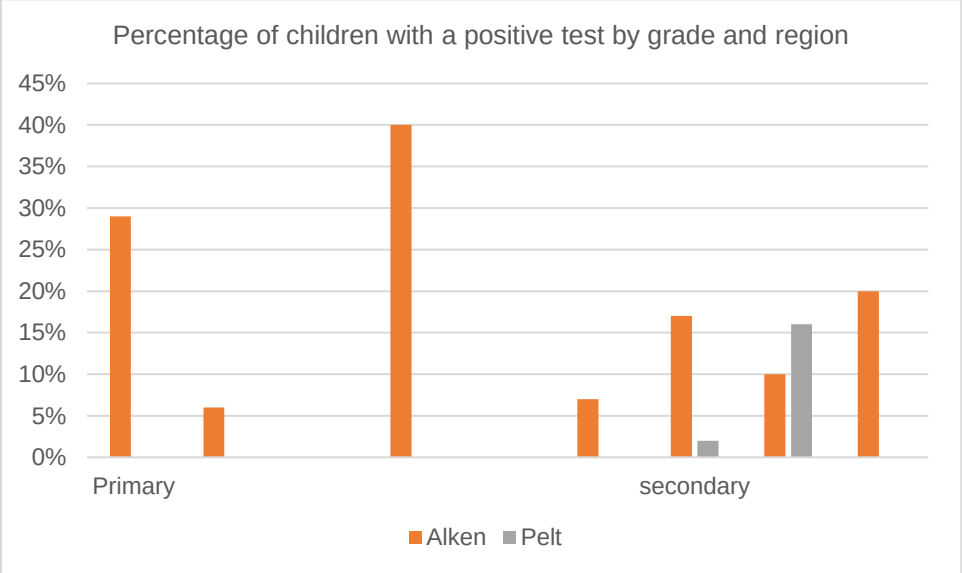


Figure 3: Percentage of children with a positive test by grade and region.

Table 6: Percentage children with a positive test by school (small numbers; maybe limit description to a range)

		Grades	Number tested	Number (%) positive
Alken	PRI1	1-6	25	7 (28%)
	PRI2	4-6	25	1 (4%)
	PRI3	1-5	40	4 (10%)
	SEC1	1st - 2nd	16	2 (12%)
	SEC2	1st - 3rd	13	3 (23%)
	SEC3	1st - 2nd	6	1 (17%)
	SEC4	1st - 3rd	56	8 (14%)
Pelt	PRI1	1-6	57	0
	PRI2	1-6	34	0
	SEC1	1st - 2nd	90	8 (9%)

For four (1.2%) children an infection with SARS-CoV-2 was reported to have been confirmed after a swab was taken in March 2020. Parents of 35 children (10.2%) were unsure if their child had been infected or not.

Table 7: Number (%) seropositive and seronegative children according to a proven previous SARS-CoV2 infection (reported by the parents;)

	Positive antibody test	Negative antibody test	Total
Past infection	4 (100%)	0	4
No past infection	22 (7.5%)	272 (92.5%)	294
Don't know	7 (16.3%)	36 (83.7%)	43

4.2.2 Background data on symptom history possibly related to increased risk of infection with SARS-CoV-2

In the questionnaire parents were also asked if their children developed symptoms that might be attributed to COVID-19 disease.

Parents of 60.1% children reported symptoms between February 2020 and sampling. This percentage increased to 87.9% for children with circulating antibodies but was still 57.1% in children without antibodies.
Since it was a retrospective study over the past six months, it is impossible to know whether the symptoms were experienced at the time of infection or at another time point.

Table 8 gives an overview of the most important symptoms that were observed by the parents since march 2020, as well as the prevalence in children with circulating antibodies (the sensitivity), the absence of symptoms in children who tested negative (the specificity), and the percentage children with a positive test in those who presented with the symptom (the positive predictive value).

Table 8: Symptoms since March 2020

Symptom	Frequency	prevalence	Se	Sp	PPV
Fever	38	11.1	21.2	89.9	18.4
Cough	88	25.8	36.4	75.3	13.6
Difficulty breathing	8	2.3	6.1	98.1	25
Loss of taste	2	0.6	6.1	100	100
Loss of smell	2	0.6	6.1	100	100
Another symptom	196	57.5	84.8	45.5	14.3
Any symptom	205	60.1	87.9	42.9	14.1

Se: percentage of seropositive children presenting with the symptom (sensitivity);
Sp: percentage of seronegative children for whom the symptom was not reported (specificity);
ppv: percentage of seropositive in children presenting with the symptom;
npv: percentage of seronegative children in those presenting without the symptom.
Other symptoms include headache, runny nose, sore throat, ear pain, myalgia, asthenia, bellyache, diarrhea, nausea, rash, hyperemia, painful eyes.

4.2.3 Factors with a possibility of influencing seropositivity

Clustering in schools/classes

Even though our study was not designed to study risk of infection within school we plotted the number of children with a seropositive test and total number of children tested in each class, ordered by grade, level and region to detect possible clustering of cases in the same class.
This plot shows limited evidence of clustering in Alken, primary (4 and 3 cases in the same class, same school); and Pelt (3 cases in the same class); The intraclass correlation (icc1) is 0.14 (95%CI; 0 – 0.30). This is the fraction of the total variance that is due to variation between clusters. A value of 0.14 is not very high (and the confidence interval includes zero, or absence of clustering), but justifies the use of cluster robust methods for the estimation of standard errors and confidence intervals (Figure 1).

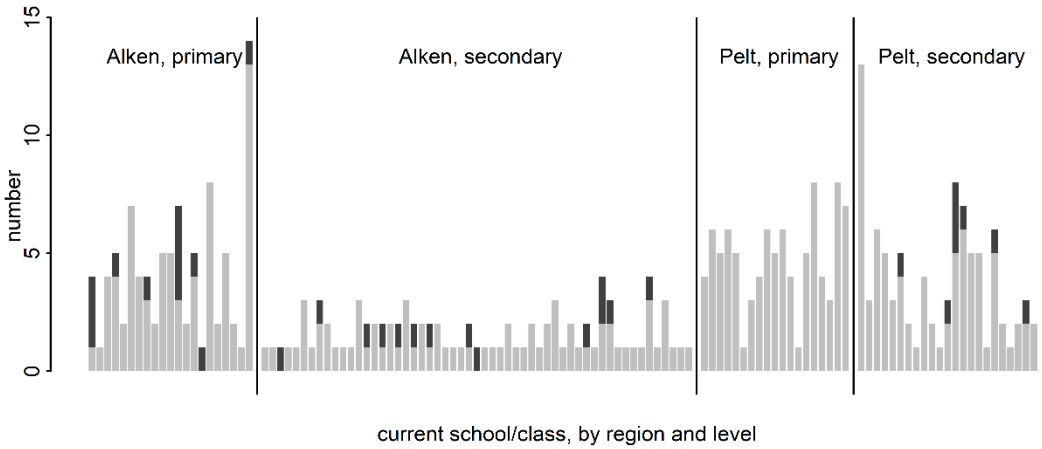


Figure 4: cases by school and class.

Contact with a confirmed case

For 41 children (12%) parents reported a contact with a confirmed case of COVID-19 (211 did not have any contact with a confirmed case, and 87 parents didn't know if the child had such a contact). Of the 41 children who had a contact, 26.8% were seropositive, which reduces to 6.6% in children who did not have a contact; and 12.6% when this was not known (chi-square test, $p < 0.001$). The relative risk of having antibodies in children who had contact with a confirmed case was 3.99 (95%CI: 1.89 – 8.41) and was 2.07 (95%CI: 0.93 – 4.58) for parents who reported that they were unsure of a possible contact.

About half (n = 19, 53.7%) of the contacts with a confirmed case were within the household (all adults, except for 1 contact with a sibling < 10 years of age), and a total of 29 (70.7%) were considered high risk contacts (no protection, < 1.5m, for > 15 minutes). All except one contact in the household were designated as high risk (n = 18), and 11 high risk contacts occurred outside the household. When the contact was a member of the household (n = 19) the prevalence of the child being seropositive is 42%; when the contact was a person outside the family, but designated as a high risk contact (no protection, < 1.5m, for > 15 minutes), the seroprevalence was 34.5%.

Public transport

In total 56 children used public transport more than once per week. Of these, 16.1% were seropositive, compared to 8.4% in those who used public transport occasionally (< 1/week). There is no statistically significant association between the presence of antibodies and the use of public transport (Chi squared test p = 0.1 unadjusted for clustering; RR 1.1 (95%CI 0.5 – 2.5); RD 0 (95%CI -0.1 – 0.1) adjusted for clustering, region and school level.

Summer camps

A majority of children attended a summer camp (n=182, 53.4%) with overnight stay, and another 62 (18.2%) a daytime only summer camp. There was no statistically significant association between these activities and the presence of antibodies (11.0% in those who attended an overnight camp, 11.3% in daytime camp, and 9.3% in those who did not attend a camp) (Fisher exact test, p = 0.7). Adjustment for class did not alter these conclusions (RR 1.6; 95%CI 0.7 – 3.5; p = 0.3 for an overnight camp, 1.8; 95%CI 0.8 – 3.9; p =0.2 for a daytime camp).

Activities outside school curriculum

Almost half of the participants had more than 3 hours/week of activities outside the school curriculum (n = 168, 49.3%), and another 122 (35.8%) between 1 and 3 hours/week. Only 11 (3.2%) children reported less than 1h/week or no (n = 40, 11.7%) activities at all.

Only one (2.0%) of the 51 children who participated in these activities less than 1h/week was seropositive, while this increased to 32 (11.0%) in the 290 children who participated in these activities

for more than 1 hour per week. This difference was statistically significant (Fisher exact test p = 0.04), also after adjustment for region, school level and clustering (RR 5.6; 95%CI 1.2 – 25.3; p = 0.03). There was no further difference for activities of more or less than 3 h/week.

5. DISCUSSION

One of the objectives of this study was to determine the SARS-CoV-2 seroprevalence in two municipalities with different background rates of confirmed SARS-CoV-2 infection.

Anti-SARS-CoV-2 antibody prevalence as indicator for past COVID-19 infection among children

IgG antibodies for SARS-Cov-2 were found in 4.4% and 14.4% in the low versus high prior exposed region, respectively.
Our study shows that if circulation in the community is high, children will also become infected. Moreover, the fact that in Alken no difference was noted between children of primary school and young adolescents in secondary schools suggests that they are equally susceptible.
In our study, children who had a contact with a confirmed case were four times more likely to have circulating antibodies compared to children for whom the parents reported no contact with a confirmed case. Children for whom parents reported uncertainty for this question where twice as likely to have circulating antibodies. The majority of these contacts in our study were household exposures. Other studies confirm the association between household exposure and circulating antibodies (Gudbjartson et al, 2020; Waterfield et al, 2020).
There is evidence that schools are not the place where circulation thrives, especially if infection control measures are applied.(Ehrhardt et al, 2020; Otte in Kampe et al, 2020;) Even though our study was not designed to study transmission in school, cluster-analysis of our data cannot exclude that transmission in schools might have occurred but if it occurred it did most probably not contribute to a large extent in the transmission of the virus. Additionally, we should not forget that infections in our population most probably took place in the first wave (see below) at a time when no corona measures (social distancing, wearing masks, ventilation of classrooms and limiting social contacts) were applied yet. In addition, our study design does not allow for analyses of the dynamics in the school and classes of last school year.

Given the assumption that the infections took place in the first wave, approximately six months prior to sampling, the antibody titers were still very robust (titer range 16.8 to 18.2 units/L) and did not show evidence of waning. There have been several publications in which waning of antibodies in persons with a prior positive PCR-test was described, and where 40% of asymptomatic individuals following confirmed infection were more likely to lose their antibodies within 3 to 6 months compared to patients who had symptoms (12.9%) (Long et al 2020). Other studies have shown limited waning in patients, such as in Iceland, where 90% of patients retained positive antibody levels up to 120 days after diagnosis.(Gudbjartson et al, 2020) Nevertheless, it cannot be ruled out that some children were infected, but lost their antibodies prior to sampling in our study. In this analysis we used the most conservative approach, favoring a high specificity above sensitivity to decrease the risk of false positive samples due to cross-reactivity.

Compared to different seroprevalence studies in Belgium (residual blood samples, healthcare workers and blood donors) the prevalence of antibodies is higher. It might be that the other seroprevalence studies underestimate the true circulation of the virus. This might be due to the specific populations from which samples are taken. Even though healthcare workers might have a higher likelihood to be in contact with persons with acute SARS-CoV-2 infections, they usually wear protective equipment to protect themselves from infection. Several studies have now shown that using face masks helps to reduce viral transmission (Ueki et al, 2020). Also, blood donors are usually persons who are more health conscious and behave as such.
Regional differences in seroprevalence have also been described in other countries (Waterfield et al, 2020, Hippich et al, 2020)

Context of the study

In this cross-sectional study, we calculated the seroprevalence in school aged children comparing the attack rate in pediatric population of a high versus low exposed municipality.
To be able to interpret the seroprevalence data correctly, and to put them into context of the evolution of the pandemic in both municipalities, we looked at number of confirmed infections and number of infections per 1000 inhabitants in Pelt and Alken (figure 5 and 6 respectively).
From these data it is clear that the virus circulated more in Alken compared to Pelt and that between the end of April 2020 and our sampling period (21 to 28 September 2020 for Pelt and 21 September to 6 October 2020 in Alken) transmission of the virus was low in both municipalities.
We also received the number of notifications of infections in both municipalities from the school health services (CLB Limburg) and between end of April and our period of sampling the number of notifications was equally low and absent in the age groups that we sampled (data not shown).

These data support the assumptions that the children with positive antibody titers were infected during the first wave of the pandemic in Belgium.

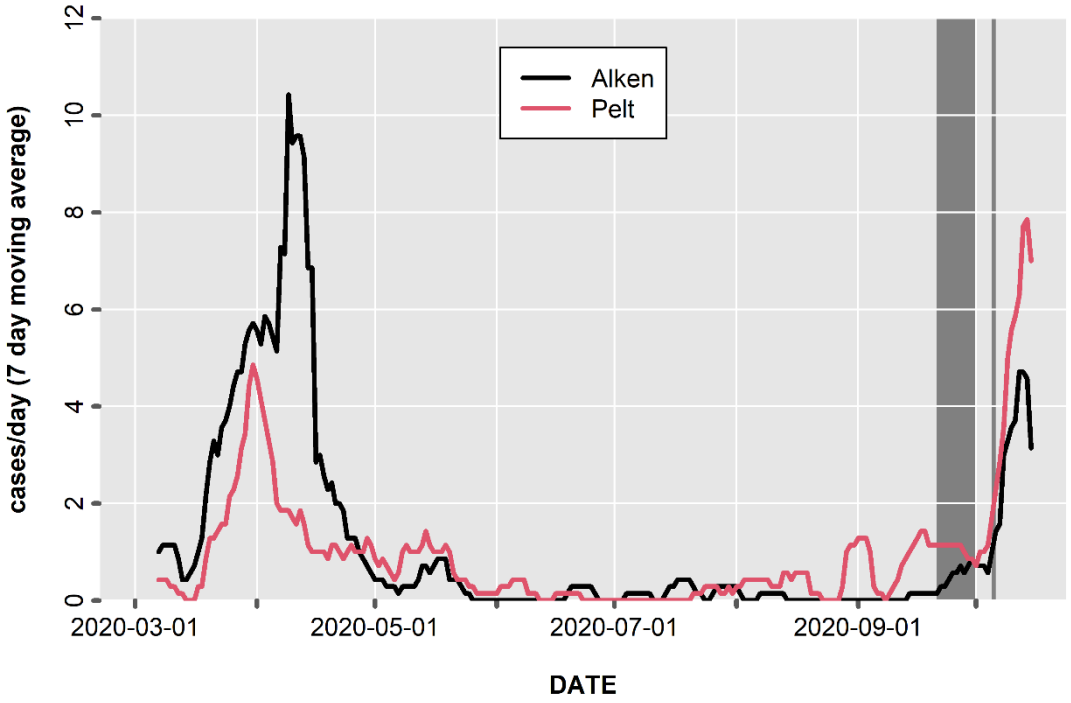


Figure 5: Number of confirmed SARS-CoV-2 infections in Pelt and Alken from March to 15 October 2020 (Data Sciensano)

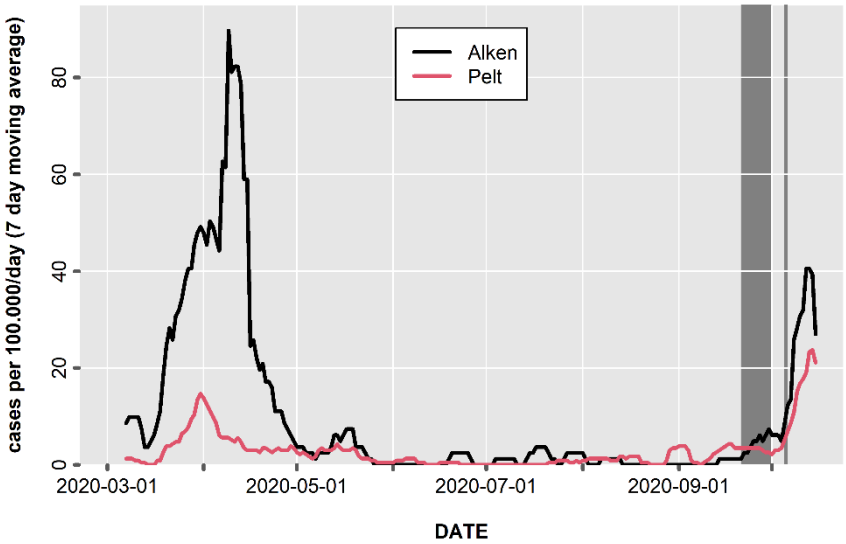


Figure 6: Number of confirmed SARS-CoV-2 infections per 1000 inhabitants in Pelt and Alken from March to 15 October 2020 (data Sciensano)

Validation of saliva sample for the detection SARS-CoV-2 antibodies

This will be reported later

Limitations of this study

This is a cross-sectional study therefore selection bias and information bias cannot be excluded. Additionally, children were not randomly selected directly into the study from a list of children residing in the municipalities; as such again selection-bias might be a problem, where only parents and children motivated to participate in the study applied for participation (e.g. family member who were sick with COVID-19 or who lost someone to COVID-19). This can lead to an overestimation of the seroprevalence of seropositivity in children. We tried to limit selection bias by the random selection of pupils of those children who enrolled for participation. In primary school children we had 390 children who enrolled for participation and in the secondary school of Pelt 150 adolescents enrolled. Only in Alken the number of adolescents who enrolled was more limited.

6. CONCLUSION

The most important findings of this study can be summarized as follows:

- Children and youngsters in Alken, where the prevalence in the community of confirmed COVID-19 disease was higher, had a higher risk to get in contact with the virus and develop circulating antibodies as confirmation of past infection. When there is increased viral circulation in a community, also children have a higher risk of getting infected.
- The risk to have a positive antibody test was higher in children who were in contact with a confirmed case, especially if this person was a household member.
- The children with circulating antibodies were most probably infected in the first wave of the pandemic in Belgium, just prior to the first lockdown on 18 March 2020.
- Children only showed mild symptoms following SARS-CoV-2 infection.
- The antibodies that were measured seems to be very robust, meaning that six months after infection antibodies in seropositive children were still very high.
- The seropositivity rate in children is higher compare to other seroprevalence studies (residual blood samples, healthcare worker study, blood donors). The other seroprevalence studies might underestimate the true circulation of the SARS-CoV-2 virus in the population.
- Infections did, most probably, not take place in school. However, one must take into account that no control measures (social distancing, wearing a face mask, washing hand frequently, limit contacts) were applicable at that time. The virus could circulate and propagate freely in this population at that time.

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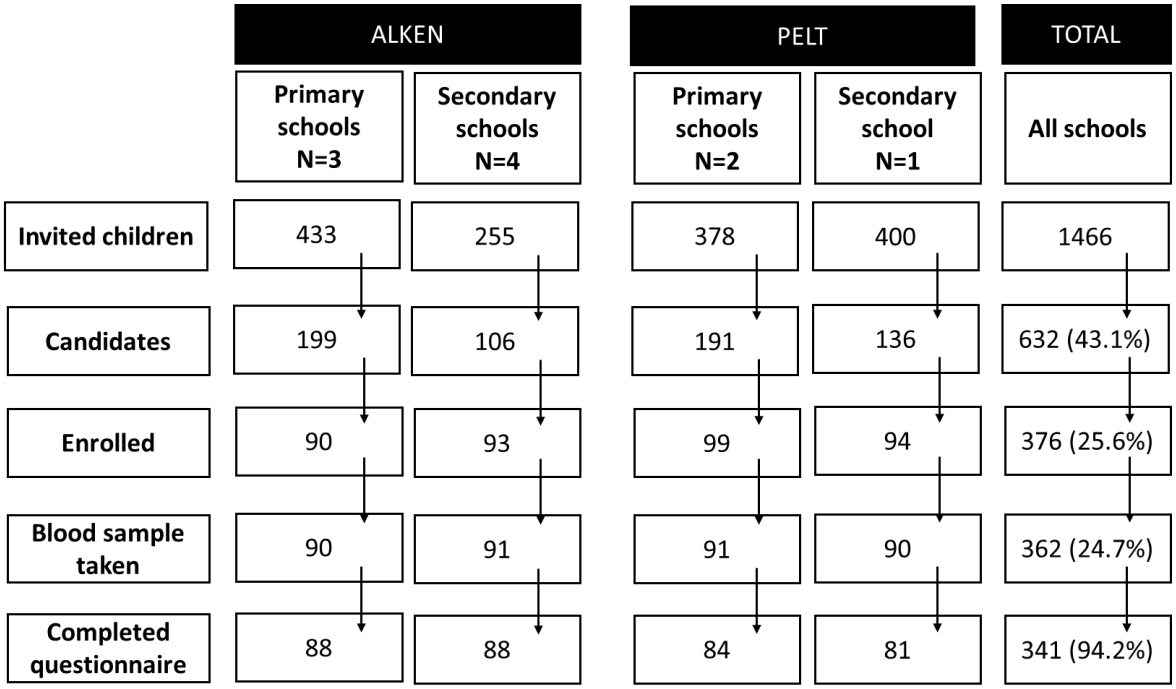
ALKEN
PELT
TOTAL
Primary schools N=3
Secondary schools N=4
Primary schools N=2
Secondary school N=1
All schools
Invited children
433
255
378
400
1466
Candidates
199
106
191
136
632 (43.1%)
Enrolled
90
93
99
94
376 (25.6%)
Blood sample taken
90
91
91
90
362 (24.7%)
Completed questionnaire
88
88
84
81
341 (94.2%)