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Title

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Running title

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Abstract & Keywords

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Recent European studies suggest an emergence of hepatitis E virus (HEV) infection. We evaluated trends in birth cohort-specific HEV seroprevalence and regional differences in Belgium. HEV IgG seroprevalence was analysed on national serum banks (1579 and 2087 samples for 2006 and 2014 respectively. Hepatitis E virus antigen was tested on positive samples. Observed data were modelled using a generalized additive model with a complementary log-log link . No significant differences between birth cohorts or sexes were found. Modelling identified the individual's age and province as relevant factors. The probability of HEV seropositivity increases significantly with age. An estimated total of 434,819 (yearly rate of 54,352) (sero-)infections was found between 2006 and 2014. Overall HEV IgG seroprevalences were 4.1% (64/1579, 95% CI 3.1-5.1) and 5.8% (121/2087, CI 4.8-6.9) in 2006 and 2014; respectively. Observed HEV antigen seroprevalence was 0.027% (1/3666) for the entire cohort. These results show stable HEV IgG seroprevalence in Belgium.

Hepatitis E, Epidemiology, Europe, Belgium

Main text

Introduction

Hepatitis E virus (HEV), a known cause for often self-limiting acute hepatitis linked to poor sanitation, feco-oral transmission and high morbidity in pregnancy, has recently come into light for numerous compelling new data. First, HEV was found to be a cause for chronic infection, mostly in solid-organ transplant recipients (1). Moreover, HEV infection is associated with a wide range of extrahepatic manifestations, such as neuralgic amyotrophy (2). The third issue is the reported high seroprevalences in regions of France, the UK and the Netherlands (3,4,5). To date, seroprevalence data for Belgium are however lacking. While HEV genotypes 1 and 2 – the causes of aforementioned epidemic hepatitis- are waterborne, genotype 3 infection is a zoonosis that may be contracted by humans through the consumption of pork and game food products or other food sources exposed to husbandry-related water or fertilizer (6). Initial European seroprevalence

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studies were fragmented and regional – with high heterogeneity in results and methodology (7). Moreover, older seroprevalence results may have been underestimations because of performance differences in commercial anti-HEV ELISA assays (8). More recent studies in Europe, based mostly on blood donors, showed an expected higher HEV seroprevalence in older populations, but also new trends. A study by Hogema et al. found that between 2000 and 2010, there was a rise in HEV IgG seroprevalence in 18-21-year-old blood donors in the Netherlands (3). In addition, a growing number of reported HEV infections in European member states were noted between 2005 and 2015 (9,10). These data have raised the question whether HEV is an emerging pathogen in Europe. Public health implications already include the institution of screening of blood donors for HEV RNA in the United Kingdom, the Netherlands, Ireland and Switzerland, although this has not been implemented more widely. Crucially however, European guidelines state that blood donor sampling inadequately represents the general population (11, 12, 13) as opposed to a well-planned, systematic sampling (14, 15). As an alternative to systematic sampling, epidemiological surveys of infectious diseases using residual sera of diagnostic laboratories have been found to yield similar results to random sampling (16).

We aimed to investigate overall HEV IgG seroprevalence and prevalence of active infection in the general population in Belgium and to detect any birth-cohort associated evolutions, using historical serum banks collected in 2006 and 2014 and mathematical modelling.

Methods and materials

The historical serum banks we used had been collected using residual samples from diagnostic laboratories (all samples collected in 2014 and up to 19-year-olds in 2006) and blood donors (adult samples collected in 2006 only). Criteria of selection was based on availability of sufficient sample volume. Only samples of which more than 50 uL was available were included. The collection design was stratified for age, gender and geographical region. Children and adolescents were oversampled in the surveys because of an initial focus on (childhood) vaccine-preventable infections (17). In order to examine an emergence of HEV infection between both sampling periods (2006 and 2014), sample size calculations were performed to select samples by birth cohort categories, depending on the number of available samples in the two surveys. A unilateral test with 80% power and 5% type-I-error was used to detect an increase in prevalence of 6% in the birth cohorts 1984-1988, 1989-1993, 1994-1998 and 1999-2003 between the 2006 and 2014 surveys (R software, package pwr) (18,19). Other birth cohorts' sampling target was set on 100 or

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maximum amount of samples available in the respective serum banks if below 100.Overall, there was an overrepresentation of tested blood samples from younger age cohorts also for this analysis. The overall age-standardised hepatitis E prevalence was assumed to be 10% in 2006 with a regular increase of the prevalence with age as observed in other studies (3, 5). Testing was performed on 1579 and 2087 samples for the samples collected in 2006 and 2014, respectively.

Wantai anti-HEV IgG ELISA assays were performed. This test showed superior performance to other commercial kits (8). Freeze-thaw experiments on 2 samples were done (performed at baseline, after 5, 10 and 15 cycles) revealing no decreased sensitivity up to 15 freeze-thawing cycles, for both serum banks. HEV IgG results equal or above 1.1 OD/cut-off were considered positive, below 1.1 as negative. Throughout the text, we will use the prefix 'sero-' to refer to the IgG positivity as a proxy of past or present infection; (sero-)infection therefore refers to a switch from IgG negativity to IgG positivity implicitly assuming that IgG levels do not decay with time since exposure. Reflex HEV antigen (Ag) testing was performed on HEV IgG positive samples, to evaluate possible acute HEV infection. HEV RNA was performed on HEV Ag positive samples using the RealStar® HEV RT-PCR Kit 2.0 (Altona Diagnostics GmbH, Hamburg, Germany). Chi-square analysis or Fisher's Exact Test was performed in SPSS 25 to compare sex, region and birth cohort proportions (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

A generalized additive model, which is a generalized linear model but with more flexibility, with a complementary log-log link is considered to model the observed seroprevalence as a function of the individual's age (a), province where the individual lives in/or where the lab is located in (prov) and birth year (b) (20):

$$\text{cloglog}(\pi(a,b,prov))=f(a,b,prov),$$

is a general presentation of a GAM where f is a smooth function and $\pi(a,b,prov)$ is the proportion of seropositive individuals of age a, in province prov and from a certain birth year b. Several submodels were considered, and model comparison (and selection) was based on (minimizing) the Akaike Information Criterion (AIC) (21). The generalized additive model shown above allows estimating the proportion of HEV susceptible individuals in the Belgian population in the years 2006 and 2014.

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The age-specific proportion HEV IgG positive individuals per province between 2006 and 2014 was derived from the final model, i.e. the model with lowest AIC value (22). In order to retrieve the age-category specific proportion of HEV IgG positivity for the whole of Belgium, the mean of the proportion of HEV IgG sero-incidence from a certain age-category was weighted by the corresponding provinces. Afterwards, the sero-incidence per age-category was obtained by multiplying the proportion of HEV IgG positivity with the population size per age-category in Belgium (taken from the 2006 StatBel demographic information) (23).

This study has been performed according to the Declaration of Helsinki. The University of Antwerp/Antwerp University Hospital Committee for Medical Ethics approved this study on 6/4/2017 by amendment (11/50/398) on approval number B300201112739.

Results

Overall HEV IgG seroprevalences were 4.1% (64/1579, CI 3.1-5.1, when standardized for province, sex and age: 4.1%, 4.1%, 4.4%, respectively) and 5.8% (121/2087, CI 4.8-6.9, when standardized for province, sex and age: 5.7%, 5.8%, 6.1%, respectively) in 2006 and 2014 (p=0.018), respectively. No significant differences between sexes were found in 2006 (p=0.603) or 2014 (p=0.942). The probability for HEV IgG positivity increased with older age. However, no significant birth cohort-specific differences were found (Table 1). These two findings suggest a cumulative increasing probability towards HEV IgG positivity with age. The significant higher HEV IgG seroprevalence observed for the 2014 survey, can be explained by a larger proportion of older persons in the population and a higher number of samples from patients born before 1979 were available for the 2014 serum bank and fully analysed. This inherently increased the overall HEV seroprevalence for 2014 compared to 2006) Also, regional (provincial) differences in HEV IgG seroprevalence were present (figure 3). One person (a 65-year old female from the 2014 survey) was HEV Ag positive, but HEV RNA negative.

Table 2 shows generalized additive models fitted to 2006 and 2014 Belgium seroprevalence data on HEV infection, with corresponding AIC values. s(a) and s(b) are smoothing functions for age and birth year. Several models have been considered and model 5, the model with the lowest AIC-value, containing age and province, is considered as the best model for the data (bold). This model does not contain a smoothing function for age, thus equal to a generalized linear model with age

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and province as covariates. Gender was considered but was not found to contribute to a fitting model. Figure 1 shows observed HEV seroprevalence data overlaid by the mathematical models.

Applying the mathematical model, figure 2 displays the estimated sero-incidence per 5-year age category for the whole of Belgium over a time span of 8 years, together with their 95% bootstrap confidence intervals. A total of 434,819 (sero-)infections was estimated for the period between 2006 and 2014. The risk of HEV (sero-)infection increases significantly with age (p-value < 0.001).

We next compared regional differences in HEV seroprevalence between both sampling periods. Figure 3 shows provincial observational seroprevalence data. Figure 4 shows the model-based proportion IgG positive between 2006 and 2014 by age in 2006 and per province. In the former HEV IgG seroprevalence for each province is colour coded, in the latter the cumulative proportion of individuals that became HEV IgG positive between 2006 and 2014 is illustrated as a function of age and stratified per province. Both approaches illustrate that geographical differences between provinces do exist. For instance, East-Flanders shows an increase in HEV seroprevalence based on the observed, raw data (Figure 3) and corresponds with the highest green line in the modelled data (Figure 4), which acknowledges a higher proportion immune over the time span of 8 years. Another example is the Province of Antwerp for which HEV seroprevalence based on raw data remained low (figure 3) and the lowest proportion of immune individuals between 2006 and 2014 is found by the model (lowest line in Figure 4).

Finally, to report on the effect of the sampling method: the serum bank of 2006 was made up out of residual samples for age categories up to 20 years old, and blood donor samples for those older than 20– while the 2014 serum bank was made up entirely out of leftover samples. We did not observe any shifts in HEV IgG seroprevalences within age cohorts up to 20 years old or older than 20 years old in the 2006 serum bank or between the age cohorts >20 in both serum banks. This does not show a sampling method bias-blood donor or hospital based- to the results and therefore allows a firm conclusion on Belgian national HEV seroprevalences in the 8 years study timeframe.

(Supplementary Figure 1)

Discussion

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In our study, we found no increase in age specific HEV seroprevalences between 2006 and 2014 in Belgium, despite a study design aimed at examining a potential increase of HEV IgG seroprevalence between the 2 sampling periods. Our survey specifically comprised a larger proportion (2168/3666, 59.1% of the total cohort) of younger age cohorts to be able to detect a rise of seroprevalence of at least 6% in these individuals largely unexposed to HEV. An increase of HEV seroprevalence of this magnitude has previously been described in Dutch blood donors aged 18-21 years and 10-fold higher symptomatic hepatitis E cases have been reported in Europe by the ECDC between 2005 and 2015 (3,9,10). Interestingly, the latter shows a (proportional) decrease of HEV cases in persons over 50 years old from 70% aged <50 years to 40% of age <50 years between 2005 and 2015 (9,10). As modelling shows that the probability for HEV IgG positivity rises significantly with age ($p < 0.001$), this strategy influenced the overall IgG seroprevalence in our study. Indeed, observed HEV IgG seroprevalence in Belgium of 4 to 6% (4.1% for 2006 and 5.8% for 2014) is lower than in the UK (13%) (4), Norway (11.4%) (24) and Germany (15.3%)(25). However, the age compositions of these studies differed from ours, with lower (UK) or no sampling performed in younger age groups (Norway and Germany). This study's approach also explains why overall found seroprevalences differ between both sampling periods, while time-specific differences are absent: the 2014 survey consisted of slightly more older age categories (Table 1).

A total of 434,819 (sero-)infections were estimated for the period between 2006 and 2014 or a yearly rate of 54,352 (sero-)infections. Interestingly, this corresponds to the aforementioned German study where two time points were investigated (1998 and 2010) and in which a declining overall seroprevalence was reported (18.6% to 15.3%). Paired sampling in over 2,500 individuals did reveal a seroconversion rate of 6.2%, corresponding to a total of 417,242 new HEV IgG cases every year. As Germany's population is roughly 8 times that of Belgium, HEV infection pressure would not be significantly different in both countries. On the other hand, we previously reported only 417 confirmed HEV cases using HEV IgM and HEV PCR analysis between 2010 and 2017 for Belgium, despite an almost 5-fold increase in confirmed symptomatic HEV cases in that period. The latter was accompanied by a similar increase in HEV suspected cases yet a stable confirmation ratio (26). With a modelled yearly rate of about 50,000 new HEV (sero-)infections in Belgium – similar to the per capita infection rate for Germany, these data thus point to a persisting significant underdiagnosis of HEV infection in Belgium.

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Our best fit mathematical model predicts observed data as a function of age and provincial location, confirming that HEV did not emerge in Belgium between both sampling periods.

Modelled data correspond with observed variations in HEV IgG seroprevalence in Belgian provinces, although the observational data was not formally powered to detect discrete and statistically significant differences. Regional differences are important in HEV seroprevalence surveys, as previous studies have shown large heterogeneity with high seroprevalence in certain regions and lower in others (4, 5, 27). Studies in France and Italy have shown areas of hyperendemicity, with HEV IgG seroprevalence in Southern France upwards of 60%, and 42.4% - 55.3% in Northern and Central Italy. Both studies associate these regional high seroprevalences with regional differences in pork and game meat consumption.

Shortcomings of our study may include, firstly, insufficient power. Although the two serum banks were sufficiently powered for birth cohort specific HEV seroprevalence evaluation, the serum banks were originally designed to survey childhood seroprevalence (17). Birth cohorts representing individuals of middle age/elderly were relatively underrepresented.

A second possible shortcoming is the use of only HEV antigen to report HEV infection rate. This assay requires less volume and is less influenced by freeze-thaw cycles than HEV IgM or HEV RNA testing. Additionally, sample volume restrictions precluded us to perform HEV IgM and PCR testing on all HEV IgG positive samples. These issues may have resulted in a low HEV antigen positivity rate: 1/185 or 0.54% (1/3666 or 0.027% of the total survey). This is much lower than the 3.4-7.5% reported in our previous study (26), however, the present study investigates mostly leftover samples (better representing a general population), while the previous study investigated samples of clinically suspected HEV cases. Further, while combining HEV IgG antibody and HEV antigen ELISA testing provides a good way to evaluate HEV infection rate (e.g. HEV antigen correlates well with HEV RNA, while HEV IgM correlation with the latter is poor), the presence of HEV antibodies lowers the S/CO (signal-to-cutoff ratio) of HEV antigen assays. This could be a second explanation of our low HEV antigen rate (28).

Conclusion

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To our knowledge, this study represents the first Belgian national HEV IgG seroprevalence study, based on birth-cohort specific sampling. No birth-cohort specific HEV IgG seroprevalence differences are observed between 2006 and 2014. Modelling shows an increased probability for HEV IgG positivity with increasing age, a regional difference within Belgium and a yearly rate of 54,352 HEV seroconversions. These combined findings show that HEV infection has not been emerging in Belgium between 2006 and 2014.

Biographical Sketch

Erwin Ho is an M.D. Microbiologist, working at the Imelda Hospital in Bonheiden, Belgium. He is also pursuing a Ph.D. at Antwerp University Hospital/Antwerp University on the natural history and epidemiology of Hepatitis B, C, D and E.

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Tables

	2006			2014			
	sample			sample			
birth cohorts	n/N	%	CI 95%	n/N	%	CI 95%	p
1948-1953	11/52	21.2	11.1-34.7	19/88	21.6	13.5-31.6	0.95
1954-1963	20/106	18.9	11.9-27.6	37/182	20.3	14.7-26.9	0.76
1964-1973	11/168	6.5	2.8-10.3	22/175	12.6	8.0-18.4	0.06

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1974-1978	3/124	2.4	0.5-6.9	9/157	5.7	2.7-10.6	0.24
1979-1983	11/277	4.0	2.0-7.0	7/169	4.1	1.6-8.3	1.00
1984-1988	3/252	1.2	0.2-3.4	6/175	3.4	1.3-7.3	0.17
1989-1993	5/254	2.0	0.6-4.5	5/176	2.8	0.9-6.5	0.56
1994-1998	0/155	0.0	0.0-2.4	6/404	1.5	0.5-3.2	0.19
1999-2003	0/125	0.0	0.0-2.9	6/403	1.5	0.5-3.2	0.21
2004-2005	0/66	0.0	0.0-5.4	2/158	1.3	0.2-4.5	1.00
TOTAL	64/1,579	4.1	3.1-5.1	121/2,087	5.8	4.8-6.9	0.02

CI 95%: 95% confidence interval, exact method

n/N: amount of HEV IgG+ve persons / total tested persons

est. individuals: estimated individuals in population HEV IgG+ve based on HEV IgG testing on sample and year-specific population data (Statbel.be)

Table 1. Observed birth-cohort specific HEV IgG seroprevalence between 2006 and 2014 in Belgium

Model components	AIC
Model 1: a + b	1285.85
Model 2: s(a) + b	1285.83
Model 3: a + s(b)	1285.83
Model 4: a	1286.39

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Model 5: a + prov	**1221.06**

Table 2. Generalized additive models fitted to 2006 and 2014 Belgium seroprevalence data on HEV infection, with corresponding AIC values . s(a) and s(b) are smoothing functions for age and birth year. In bold: the model with the lowest AIC-value.

Figure titles and legends

Figure 1. Observed HEV IgG seroprevalence in 2006 and 2014 in Belgium with fitted mathematical model

Red and blue circles represent HEV IgG seroprevalence (0-1.00) according to age (0-65). Circle diameter represents the number of samples per age.

Lines represent mathematical modelling for 2006 (light grey) and 2014 (dark grey) respectively. Light and dark grey shading represents 95% confidence interval for both 2006 and 2014 modelled HEV IgG seroprevalences

Figure 2. Estimated sero-incidence per 5 year age-category for whole Belgium over a time span of 8 years together with their 95% bootstrap confidence intervals

Sero-incidence for whole Belgium is derived from the mean of the proportion HEV IgG positive from a certain age-category weighted by the corresponding province, multiplying with population size per age-category in Belgium.

Figure 3. Observed regional (provincial) seroprevalence of HEV IgG in 2006 and in 2014 in Belgium

Colour shading represents measured HEV IgG seroprevalence per province: 0-14.99 as shown on the colour map. Tables next to geographical map show HEV IgG positive samples, totals, percentages and respective 95% confidence intervals (lower and upper boundaries) m

Figure 4. Model-based proportion positive between 2006 and 2014 by age in 2006 per province

Lines constitute the increase in the proportion HEV IgG positive, between 2006 and 2014 by age in 2006 and per province, derived by subtracting the predicted values for age a from the best model with the predicted values when age was set to a+8 (8 = 2014 – 2006)

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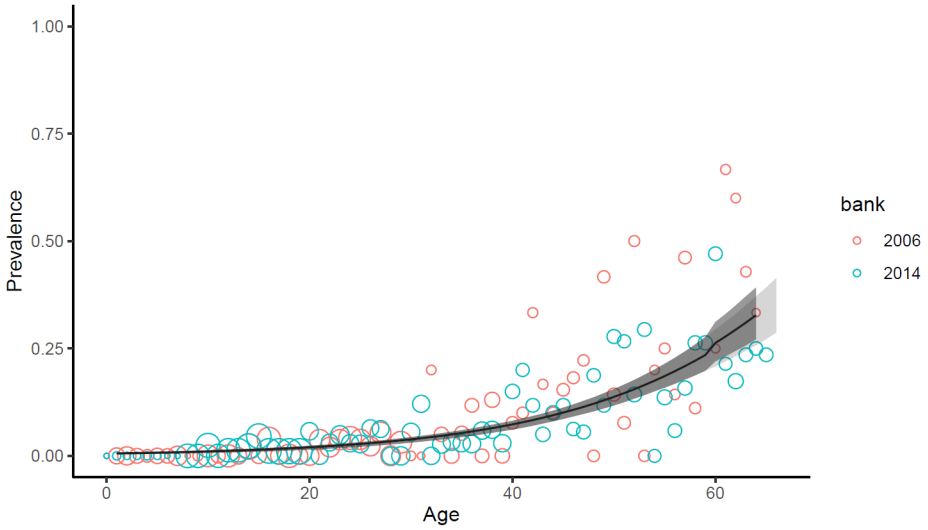

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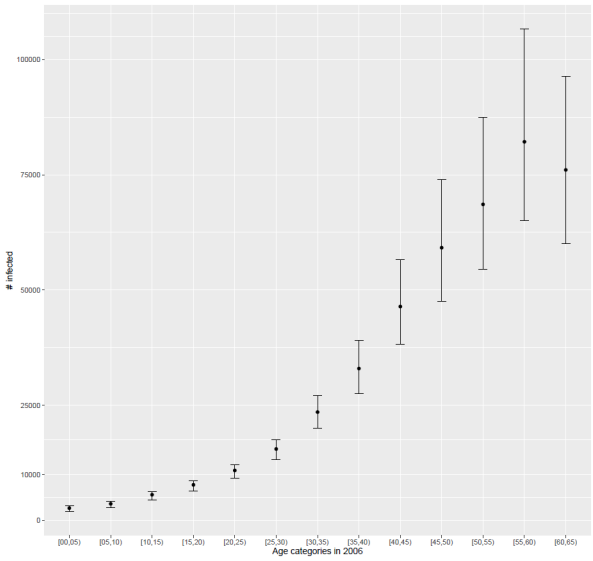

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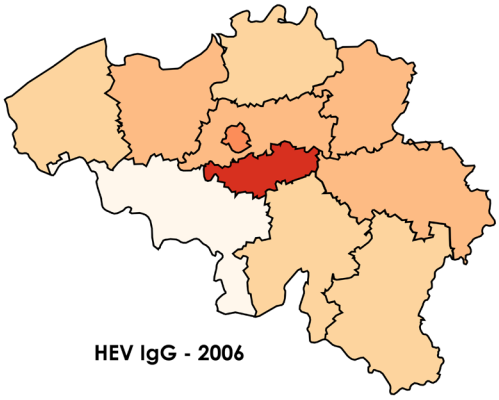
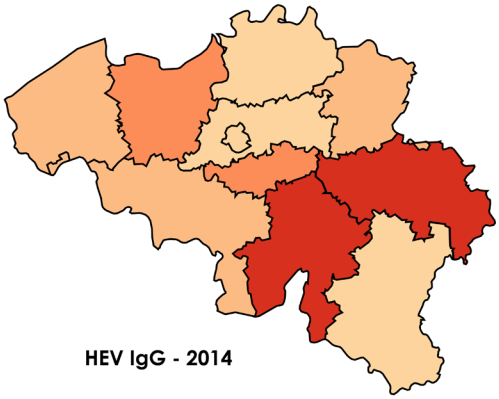


HEV IgG (province) 2006	n	N	%	CI-	CI+
Antwerp	9	236	3.81	1.76	7.12
Brussels-Capital Region	9	108	8.33	3.88	15.23
East Flanders	16	275	5.82	3.36	9.28
Flemish Brabant	7	107	6.54	2.67	13.02
Hainaut	3	197	1.52	0.32	4.39
Liege	10	190	5.26	2.55	9.47
Limburg	4	59	6.78	1.88	16.46
Luxembourg	2	58	3.45	0.42	11.91
Namur	4	106	3.77	1.04	9.38
Walloon Brabant	4	35	11.43	3.20	26.74
West Flanders	8	184	4.35	1.90	8.39

0-2.99
3.00-4.99
5.00-6.99
7.00-8.99
9.00-14.99



HEV IgG (province) 2014	n	N	%	CI-	CI+
Antwerp	11	341	3.23	1.62	5.70
Brussels-Capital Region	9	183	4.92	2.27	9.13
East Flanders	20	254	7.87	4.88	11.90
Flemish Brabant	3	94	3.19	0.66	9.04
Hainaut	10	168	5.95	2.89	10.67
Liege	12	109	11.01	5.82	18.44
Limburg	14	220	6.36	3.52	10.45
Luxembourg	5	103	4.85	1.59	10.97
Namur	5	34	14.71	4.95	31.06
Walloon Brabant	3	39	7.69	1.62	20.87
West Flanders	19	331	5.74	3.49	8.82

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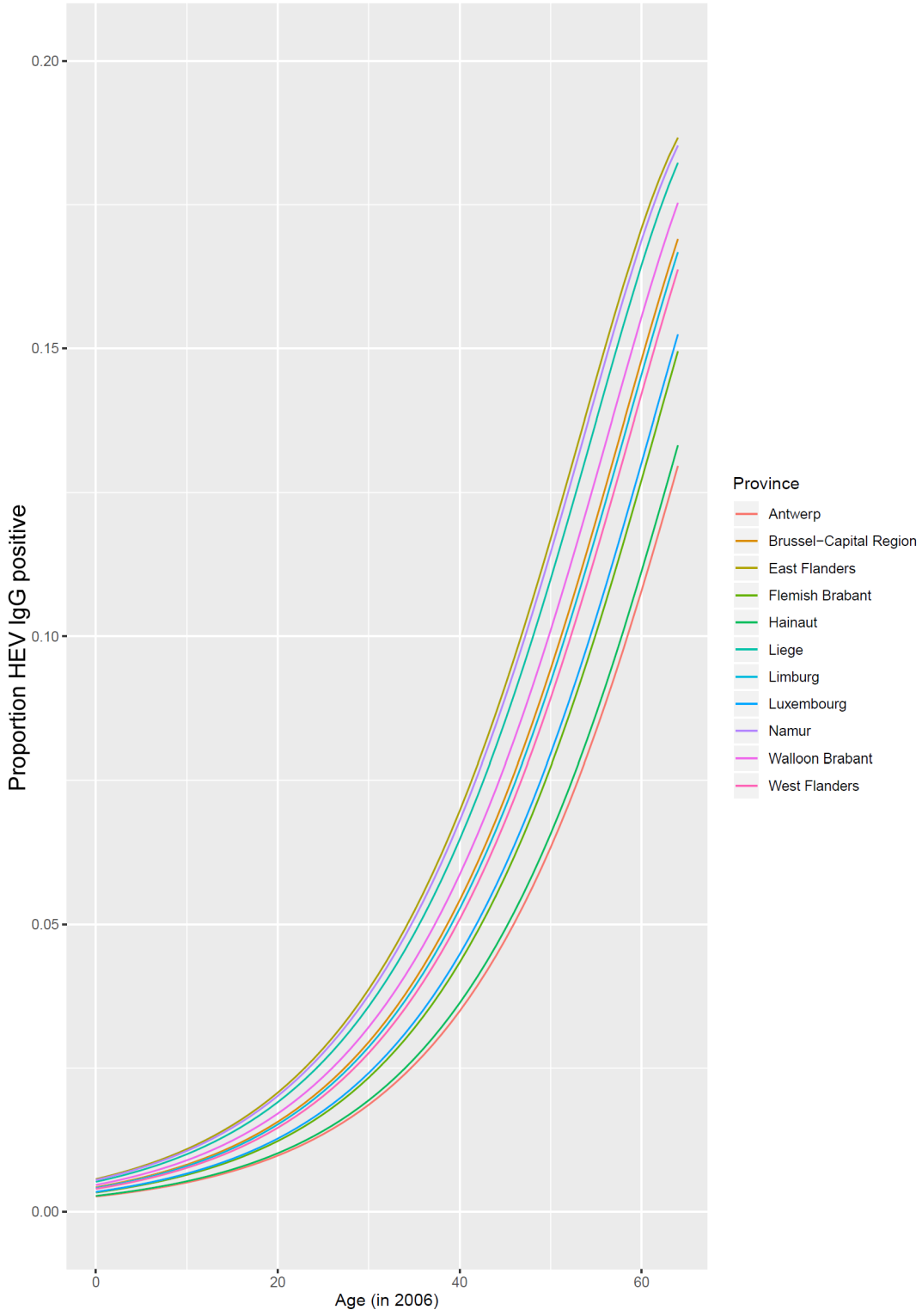

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