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Carriage of a new epidemic strain of *Neisseria meningitidis* and its relationship with the incidence of meningococcal disease in Galicia, Spain

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SUMMARY

In Galicia, Spain, a dramatic increase in the incidence of meningococcal disease was seen in the 1995–6. The annual incidence rose to 11 per 10^5 inhabitants, and 80% of identified strains were C:2b:P1.2,5. This led to the implementation of an intensive A+C vaccination campaign for the population aged 18 months to 19 years. During this campaign the prevalence of carriage in areas with high and low incidence was studied. Nasopharyngeal swabs were taken from 9796 subjects immediately before the administration of meningococcal vaccine, plated onto Thayer–Martin plates, incubated and sent for analysis to the Reference Laboratory for *Neisseria* in Spain. The prevalence of the C:2b:P1.2,5 strains was 0·6% (95% CI 0·29–0·88) in the high incidence area, and 0·41% (95% CI 0·00–1·04) in the low incidence area, and that of serogroup C (all strains) 1·36% (95% CI 0·80–1·80) and 0·89% (95% CI 0·09–1·69) respectively. The prevalence of *N. meningitidis* (all strains) was almost the same in both areas (8%). Carriers of the epidemic strain were not found in the 2–4 year age group, that most affected by the disease.

Our data showed a wide distribution but a low carriage rate of the epidemic strain C:2b:P1.2,5 in the high and low disease incidence areas studied; the difference in the carriage rates between the two areas was not statistically significant.

INTRODUCTION

Meningococcal disease (MD) incidence rose in Galicia, a region with 2·7 million inhabitants in the north-west Spain, between October 1995 and October 1996. During this time 306 suspected cases were ascertained, a rate of 11·26 cases per 10^5 inhabitants, more than three times greater than that of the preceding period 1994–5 during which 99 cases were notified (3·65 cases per 10^5 inhabitants) [1]. In the period 1995–6 *Neisseria meningitidis* was isolated from 50% of cases and of these 80% were of serogroup C, and almost all of phenotype C:2b:P1.2,5 [2]. These findings represented a change in the usual pattern of the disease given that in Galicia, as well as in the rest of Spain, the predominant serogroup until then had been serogroup B [3–5]. The new epidemic strain was isolated for the first time in March 1995 during an investigation of an outbreak in the town of Caldas de Reis.

The distribution of MD was not uniform throughout the region. This allowed us to divide Galicia into two parts: one corresponding to the urban, coastal

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fringe with a high incidence of disease and the other, a more rural, inland zone with a clearly lower incidence.

Having analysed the available data from the disease active surveillance system and made an estimate of the expected number of cases for the subsequent period (1996–7) [6] a number of possible intervention strategies for Galicia were formally evaluated using a decision-making methodology [7]. The final decision was to recommend meningococcal A + C vaccination for the Galician population between the ages of 18 months and 19 years, inclusively.

Asymptomatic human carriers of *N. meningitidis* are the only known reservoirs of the organism and represent the source of more than 95% of all invasive infections [8, 9]. The study of this group is helpful in understanding both the epidemiological patterns and the pathogenesis of meningococcal infection. Several studies have been carried out in different settings to determine the prevalence and duration of the carrier state [10–16], and the risk factors that contribute to meningococcal acquisition [17–21]. However, less has been published regarding the change in the causative serogroup of MD [22] or the prevalence of carriers in areas with different incidences of MD [23–25] in relation to the circulation of 'epidemic strains' as opposed to the circulation of *N. meningitidis* in general.

We implemented a carrier survey in the general population of Galicia between 2 and 19 years of age at the same time as the meningococcal A + C vaccine was offered, with the purpose of determining the prevalence of different meningococcal serogroups, and specifically of the strain C:2b:P1.2,5, the main cause of MD at that time, and its distribution in two areas of Galicia which were very different in terms of the incidence of disease.

MATERIALS AND METHODS

Populations and samples

At the time the study was performed (December 1996–January 1997) Galicia could be divided into two clearly differentiated zones according to the incidence of MD in 1995–6: one with high incidence (epidemic state) and the other with low incidence (non-epidemic state). Each one was made up of several administrative areas; we selected one with a gross rate of MD of 23·01 cases per 10^5 inhabitants from the high incidence area (HI), and another with a rate of 1·97 cases per 10^5 inhabitants from the low incidence area (LI). The studied populations comprised children and adolescents aged 2–19 years resident in these areas (HI and LI) and the study exploited the vaccination centres in which meningococcal A + C vaccine was administered. The specific annual rates of MD in people 2–19 years old were 76·44 cases per 10^5 inhabitants in HI area and 9·52 cases per 10^5 in LI area.

In both areas, individuals were selected using a two-stage sample design, with stratification of the vaccination centres for the population, which were the first-stage units. The secondary units comprised the population between 2 and 19 years old, who would visit the vaccination centres. The stratification of the vaccination centres was performed according to geographic criteria: two strata were established in each of the areas of incidence. The vaccination centres in each stratum were selected randomly and with a probability proportional to their size, ie to the number of people in the 2–19 population group. The final selection of individuals was carried out in a consecutive manner, according to the order in which they arrived to the vaccination centre.

The sample size was calculated in order to achieve an absolute error no larger than 0·3% in HI area nor 0·1% in LI area. Estimated prevalence of the strain C:2b:P1.2,5 of 1% in HI and 0·2% in LI were used, resulting in a sample of 9829 individuals, 4533 from the HI area and 5296 from the LI area. The age distribution of those sampled was proportional to the size of each of the following age groups: 2–4, 5–9, 10–14 and 15–19 years in the general population, as projected by the Galician Institute of Statistics (IGE) [26] for the year 1996 for these areas.

Collection of samples and individual data

The study design consisted of a transverse section, was anonymous and without the possibility of relating data to individuals. Data were collected on age, gender and area of residence of all participants. Nasopharyngeal samples were taken using sterile swabs, immediately prior to the administration of meningococcal vaccine by trained health care personnel.

Isolation of strains from carriers
Pharyngeal swab cultures and strain identification

Nasopharyngeal swabs were plated immediately onto Thayer–Martin agar plates (Oxoid UK) and sent to regional laboratories in less than 3 h, to be incubated

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for 24 h at 37 °C in 5% CO_2. Subsequently all plates were sent daily to the National Reference Laboratory (NRL) for the identification and characterization of *N. lactamica* and *N. meningitidis* by initial reading of the plates followed by at least 24 h of further incubation.

When the morphology and Gram stains were suggestive of *Neisseria* spp. colonies were replated on Thayer–Martin agar plates for purity. The production of oxidase and catalase, sugar utilization test in CTA medium (Difco, USA) and MHBT medium and investigation for β-galactosidase activity were carried out as previously described [27].

Meningococcal serogrouping and sero/subtyping

The serogroup of all meningococcal strains was determined by slide agglutination. The polyclonal sera used were produced in the NRL by inoculating rabbits following a protocol described elsewhere [27].

The serotype and serosubtype were analysed in serogroup C meningococci to identify the epidemic strain. Sero/subtype determinations were done by a whole cell enzyme-linked immunoassay (EIA). Antigens were prepared as described by Abdillahi and Poolman [28]. Monoclonal antibodies with serotype specificities 1, 2a, 2b, 4, 14, 15 and subtype specificities P1.1, P1.2, P1.3, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.13, P1.14, P1.15 and P1.16 were supplied by the National Institute for Biological Standards and Control, United Kingdom.

Culture, DNA preparation and PFGE assay

All the strains characterized as C:2b:P1.2,5 were analysed by pulsed field gel electrophoresis (PFGE). The conditions for culture of strains, DNA preparation and PFGE assay have been described previously [29].

Incidence of the disease

Although it has been estimated that in Europe the median duration of the carrier state for meningococcus is 9–10 months, serogroup C may be characterized by a shorter duration of carriage and a higher acquisition rate [30]. Therefore, to study the incidence of the disease during the carrier period we took the 4 months prior to the starting of sample collection as the average duration of the carrier state, which was the time indicated by Gold and colleagues [12]. To avoid the effect that vaccination could have on the disease incidence the period subsequent to sample collection was not included [31].

Statistical analysis

Data for each area was analysed separately, using the unbiased design-based estimator for the population prevalence $\hat{P} = \hat{X}/M$, where: M is the population size, $\hat{X}$ is the estimator for the total population of carriers, given by:

$$\hat{X} = \sum_{h=1}^{2} \sum_{i=1}^{n_h} w_{hi} x_{hi},$$

where h denotes the stratum and i the ith primary sampling unit (vaccination centre); n_h is the number of sampled vaccination centres within stratum h, x_{hi} is the total sample of carriers in the ith centre and w_{hi} is the sampling weight, given by:

$$w_{hi} = \frac{1}{f_{hi1}} \frac{1}{f_{hi2}} = \frac{M_h}{n_h m_{hi}},$$

where $f_{hi1} = n_h(M_{hi}/M_h)$ is the first stage sampling fraction and $f_{hi2} = (m_{hi}/M_{hi})$ the second stage sampling fraction; m_{hi} is the number of samples collected in the ith centre.

The variance estimator for the estimated prevalence was calculated as:

$$\hat{V}(\hat{P}) = \frac{1}{M^2} \sum_{h=1}^{2} \frac{n_h}{n_h - 1} \sum_{i=1}^{n_h} (y_{hi} - \bar{y}_h)^2,$$

where:

$$y_{hi} = w_{hi} x_{hi} \quad \text{and} \quad \bar{y}_h = \frac{1}{n_h} \sum_{i=1}^{n_h} y_{hi}.$$

Approximate 95% confidence intervals for the unknown population prevalences were obtained using a normal distribution.

Data to derive the estimated prevalences and their variances from these formulae are included in Appendix 1 and 2 for high and low incidence areas, respectively.

RESULTS

A total of 9796 nasopharyngeal samples were obtained, 99·5% of those planned. Of these, 4562 were from the HI area population and 5234 from the LI

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Table 1. Neisseria *carriage rates in each area**

	High incidence			Low incidence		
	n	%	95% CI	*n*	%	95% CI
All samples	4490			5234		
All Neisserias	659	13·94	12·01–15·86	708	14·08	12·26–15·91
All *N. meningitidis*	401	8·78	7·12–10·44	391	8·03	6·71–9·34
Serogroup C	55	1·36	0·85–1·86	32	0·89	0·09–1·69
C:2b:P1.2,5	31	0·60	0·28–0·93	10	0·41	0·00–1·04
Serogroup B	260	5·94	4·50–7·30	262	5·15	4·30–5·90
Other serogroups	13	0·25	0·07–0·04	14	0·44	0·00–0·89
Non groupables	73	1·22	0·79–1·65	83	1·55	0·98–2·12
N. lactamica	254	5·16	3·50–6·70	308	6·06	4·90–7·10
Other *N* species	4	0·10	0·00–0·24	9	0·15	0·02–0·27

* Percentages are calculated on the basis of a two-stage sampling procedure (see Statistical analysis), and are therefore different from these that would result if calculations had been based on a one-stage sampling procedure.

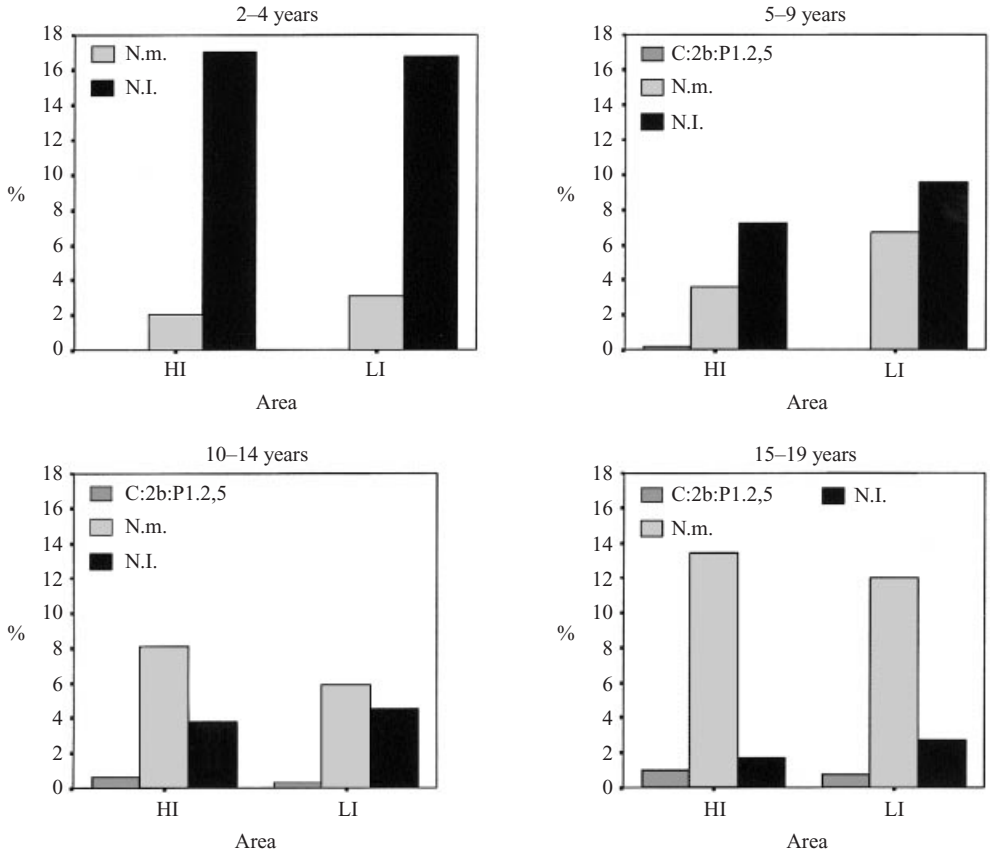

Fig. 1. Neisseria carriage rates by age group and area.

area population. In the HI area 47·4% of the samples collected were from males, and 50·0% from females; in 2·7% of cases the gender was not documented. In the samples collected in the LI area the gender was not registered in 0·5% of cases, the rest of the sample being evenly distributed between males and females.

Seventy-two samples taken from the HI area were excluded from the analysis because their first-stage unit was not known. Only two of these samples yielded the epidemic strain C:2b:P1.2,5.

N. meningitidis serogroup C was isolated from 87 samples and 27 different phenotypes were found. The

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Table 2. *Cases of serogroup C meningococcal disease and carriages rates of epidemic strain by age and area*

Age group (years)	Area	Cases serogroup C	Carriage of strain C:2b:P1.2,5
2–4	HI*	3	0·00
	LI†	0	0·00
5–9	HI	2	0·19
	LI	0	0·00
10–14	HI	0	0·62
	LI	0	0·33
15–19	HI	2	1·06
	LI	0	0·79

* High incidence
† Low incidence

epidemic strain C:2b:P1.2,5 was present in 41 samples (47·1% of the total of serogroup C); 31 of these were from HI and 10 from LI. Analysis with PFGE showed a total absence of genetic diversity in the 41 strains of C:2b:P1.2,5.

The prevalence of the epidemic strain C:2b:P1.2,5 was 0·6% in HI area and 0·41% in LI area. Although the prevalence was higher in HI, the difference was not statistically significant.

Similarly, the prevalence of carriers of *N. meningitidis* serogroup C in the HI area (1·36%) and in the LI area (0·89%) did not show a statistically significant difference. However the prevalence of *N. meningitidis* serogroup B (Table 1) was significantly greater than that of serogroup C in both areas studied. The 95% confidence intervals for the difference between the two serogroups in the HI area was 0·03–0·06 ($P < 0·0001$) and in the LI area 0·03–0·05 ($P < 0·0001$).

The overall prevalence of carriers of *N. meningitidis* and *N. lactamica* was 13·94% in the HI area and 14·08% in the LI area. Percentages according to species and serogroup of *Neisseria* are presented in Table 1. We did not identify carriers of *N. meningitidis* serogroup A or W135.

The prevalence of carriage increased progressively with age in both areas, although no statistically significant difference was found between areas nor between age groups within the same area. The distribution of carriage by age group and area is presented in Figure 1. In the 2–4 year age group we did not find carriers of the epidemic strain in either of the two studied areas, and in this age group only 0·24% of carriers of serogroup C were observed in the HI area. The typical, progressive decrease in *N. lactamica* and a corresponding increase in *N. meningitidis* carriage with age was seen.

No statistically significant difference was found in the prevalence of *N. meningitidis* serogroup C by gender between the two study areas. There was a statistically significant difference between males and females in the LI area with regard to serogroup B.

During the 4 months prior to the study we detected seven cases of *N. meningitidis* serogroup C infection, all of them in the HI area, an area in which the population was double the size of that of the LI area. In Table 2 the distribution of those seven cases is shown (assumed to be due to the epidemic strain C:2b:P1.2,5) by age group as well the carriage rates of the epidemic strain.

DISCUSSION

This is the largest study of meningococcal carriage to be reported to date (9796 samples). It facilitated a good approximation of the distribution of *N. lactamica* and *N. meningitidis*, of serogroups and of the epidemic strain stratified by age group and gender. Furthermore, the large sample size also facilitated a close approximation to the relationship that might exist between the prevalence of carriers and the incidence of meningococcal disease in the general population and not just to the population affected in an outbreak.

Studies of the prevalence of carriers of *N. meningitidis* carried out in Spain in the general population are few [33–35] and none of these analysed the possible differences, within the same geographic region, between areas with a different incidence of disease.

The kind of sampling design implemented for the selection process, a two-stage sample design, with stratification of the first-stage units, demanded a rather complex statistical management of the results. To estimate the carriage rates in the population, the formulae needed to take into account the corresponding first-stage units, in order to permit the application of the appropriate weighted estimators to both stage and area.

Because of this, we could not include in the analysis the sampling units for which the first-stage unit was not registered. As a consequence, two samples of the epidemic strain C:2b:P1.2,5 taken from the HI area were not included in the analysis.

The overall proportion of carriers of *N. meningitidis* was almost identical in the study areas (8·78% in HI

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and 8·03% in LI) and was lower than that found (12·7%) in a population in the province of Barcelona [34] in the north-east of Spain. However, the prevalence we detected in the 6–14 age group (6·40% in HI and 6·37% in LI) was higher than that found in a study carried out in a school population in the Madrid area [33].

In some studies carried out in other countries either in closed populations [13, 36, 37] or during an outbreak [14], the prevalence of *N. meningitidis* was greater than that we detected in general population. Of the studies reviewed where the prevalence of carriers of *N. meningitidis* and *N. lactamica* was determined in areas with high and low incidences of MD, the one carried out in the Faroe Islands [24] during a hyperendemic situation due to serogroup B gave a prevalence of *N. meningitidis* higher than ours, with statistically significant differences between the areas in their study.

A Danish study [23] compared the prevalence of *N. meningitidis* serogroup C in two areas, one of 'high risk' (where cases of MD due to serogroup C had occurred) and another of 'normal risk' (where cases had not occurred) during an outbreak of *N. meningitidis* serogroup C in adolescents (aged 16–20). The prevalence of carriers of *N. meningitidis* and of serogroup C found in this study [23] were 30 and 3% respectively, the same in both areas, and much higher than those we found in either study area in the 15–19 age group.

However, in a Canadian study [25], again areas with different incidence rates for MD, the carriage rate of *N. meningitidis* found was lower than ours. In this study, the authors found statistically significant differences in the rate of carriers between areas of high and low incidence when the two areas were distant from each other (3500 km apart), but not when the two areas were closer geographically (270 km apart). In our case, the areas most geographically separated were not more than 250 km apart. Equally, in the study of Stonehouse, English [14], the 'outbreak strains' were found more frequently in areas with a high disease incidence, but there were areas of the same town equally colonized by the same type of meningococcus.

We found no statistically significant difference in the prevalence of carriage of the epidemic strain C:2b:P1.2,5 between HI and LI areas agreeing with the results of Ronne and colleagues in their study (C:2a:P1.2 sulphonamide-resistant strain [23].

As we expected to find a statistically significant difference in the rates of carriage of the epidemic strain C:2b:P1.2,5 between both areas we carried out a detailed study of our epidemic strains according to sampling location and we checked that in the LI area all the sampling locations except one, showed a carrier prevalence between 0 and 0·7%. This other location showed a prevalence of 3·53%, much higher, even, than the highest prevalence of the high incidence area (1·92%). Given that the estimators used in the study design were very sensitive to outliers, this point had a great influence on the overall prevalence of its area, in such a way that the difference between the prevalence of the strain in both areas was not statistically significant.

It should be stressed that, even if this point had showed a prevalence similar to that observed in the high incidence area, the overall prevalence of carriers of the strain C:2b:P1.2,5 in the LI area would have shown a statistically significant difference from that found in the HI area.

The finding of high prevalences of carriers within small groups, within a defined area, is compatible with the epidemiology known for meningococcal infection. Since our sampling was followed immediately by vaccination of almost 100% of the at risk population, we would not exclude the possibility that the relatively high carriage rate of the epidemic strain might be related to an outbreak of disease. The hypothesis that there may be a relationship between the prevalence of carriers of the strain and the incidence of the disease in each of the study areas cannot be ruled out.

Regarding the number of isolates within the same phenotype, the percentage was similar in both our study and the experience of Le Saux and colleagues [25] who found three isolates with the same phenotype (2a:P1.2) of the strain of serogroup C responsible for the increase in the number of cases of MD. Of these three, none was of the same clone as the strains isolated from affected patients. In our study, the 43 C:2b:P1.2,5 strains were found to be genetically homogeneous by PFGE and multilocus enzyme electrophoresis (MLEE), and to belong to the same clone as that isolated from affected patients (data not shown).

The low carriage rates of the epidemic strain, the fact that in the age group most affected by the disease (2–4 years) we did not find carriers of the epidemic strain and, within this age group, the carriers of *N. meningitidis* serogroup C were only found in the HI area, as well as the fact that serogroup B continued to be the most prevalent serogroup in all age groups and

in both study areas, all suggest an important degree of pathogenicity for strain C:2b:P1.2,5.

The change in the epidemiological pattern of MD presented in Galicia from 1995 onwards manifested as a high incidence of disease due to serogroup C, phenotype 2b:P1.2,5, did not correspond with a change in the serogroup distribution amongst carriers of *N. meningitidis*, which continued to be predominantly B strains, as has been the case in Spain, as a whole, in the last few decades [33, 35, 38, 39].

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Appendix 1. Data to calculate the estimated carriage rates of High Incidence area

Stratum	Centre	m_{hi}	Weight	All *N.*	All *N.m.*	Serogroup C	C:2b:P1.2,5	Serogroup B	Other	Non-groupable	*N. lactamica*	Other *N.*
1	1-001	248	28·1418018341064	21	14	5	3	7	1	1	7	2
	1-002	461	15·1391897201538	61	35	8	4	20	0	7	26	0
	1-003	266	26·2374687194824	33	8	3	2	4	1	0	25	0
	1-004	365	19·1210041046142	41	25	7	7	15	1	2	16	0
	1-005	205	34·0447158813476	38	20	2	0	16	0	2	18	0
	1-006	25	279·1666564941400	3	3	1	0	2	0	0	0	0
2	2-001	527	11·3465366363525	84	51	10	4	24	1	16	33	1
	2-002	357	16·7496490478515	59	40	4	2	29	0	7	19	0
	2-003	327	18·2863159179687	61	40	3	2	27	4	6	21	0
	2-004	56	106·7790145874020	6	5	0	0	5	0	0	1	0
	2-005	463	12·9149570465087	95	64	4	2	45	3	12	31	0
	2-006	489	12·2282724380493	69	51	6	4	35	1	9	18	0
	2-007	387	15·4512271881103	45	21	1	0	13	0	7	24	0
	2-008	314	19·0433921813964	39	24	1	1	18	1	4	15	1

Stratum	n_h	M_h
1	6	41875
2	6	47837

Appendix 2. Data to calculate the estimated carriage rates of Low Incidence area

Stratum	Centre	m_{hi}	Weight	All *N.*	All *N.m.*	Serogroup C	C:2b:P1.2,5	Serogroup B	Other	Non-groupable	*N. lactamica*	Other *N.*
1	3-001	181	13·7857581338244	30	15	0	0	9	0	6	15	0
	3-002	604	4·1311626195732	76	51	4	0	30	2	15	25	1
	3-003	666	3·7465799132466	71	41	0	0	33	1	7	30	1
	3-004	521	4·7892940925571	97	48	7	2	27	0	14	49	1
	3-005	437	5·7098906687007	49	32	3	1	22	1	6	17	0
	3-006	496	5·0306899641577	63	28	2	1	15	2	9	35	3
	3-007	530	4·7079664570231	90	50	3	0	35	1	11	40	0
	3-008	523	4·7709793923943	73	43	2	1	35	1	5	30	1
	3-009	85	29·3555555555556	16	11	4	3	5	2	0	5	0
2	4-001	286	8·7245532245532	30	16	3	2	11	0	2	14	0
	4-002	165	4·7045454545455	16	11	0	0	10	0	1	5	2
	4-003	92	8·4375000000000	15	5	2	0	3	0	0	10	0
	4-004	405	1·9166666666667	43	22	1	0	15	1	5	21	0
	4-005	47	16·5159574468085	2	0	0	0	0	0	0	2	0
	4-006	82	9·4664634146342	12	8	1	0	4	3	0	4	0
	4-007	36	21·5625000000000	6	5	0	0	4	0	1	1	0
	4-008	78	9·9519230769231	10	5	0	0	4	0	1	5	0

Stratum	n_h	M_h
1	9	22457
2	8	6210