Temporary negative results of serological monitoring of rats with enzootic *Haemophilus* infection

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Summary

Infection by V-factor requiring *Pasteurellaceae* (*Haemophilus* spp) bacteria frequently occurs in Specified Pathogen Free (SPF) colonies of rats. Colonies may be monitored by methods that either detect the bacteria or detect specific antibodies by for instance the enzyme-linked immunosorbent assay (ELISA) and immunoblot (IB).

Breeding females in enzootically infected colonies transfer antibodies to their young. Maternal immunity prevents the development of antibodies by the pups yielding a transition period of low antibody activity in the pups between waning maternal immunity and seroconversion.

We studied antibody activity to *Haemophilus* antigens in young rats delivered by *Haemophilus* infected dams by ELISA and IB using antigens of *Haemophilus* strains H21 (from rat) and H35 (from guinea pig).

Young showed high ELISA and IB maternal antibody activity until weaning, a decline to a low level between 9 and 12 weeks of age, and then seroconversion.

As a consequence seroprevalence varied with age and during the transition period H21 ELISA yielded a false negative result in *Haemophilus* infected rats.

The age-related variation in antibody activity in enzootically infected rodents has an impact on the development of sampling strategies for serological health monitoring and interpretation of its results.

The Federation of Laboratory Animal Science Associations (FELASA) recommends monitoring rodent colonies for all Pasteurellaceae bacteria (*Nicklas et al., 2002*). Pasteurellaceae comprise growth factor independent species such as *Pasteurella pneumotropica* and bacteria that need X- (hemin) Jand/or V-factor (nicotinamide adenine dinucleotide [NAD]) for growth (*Olsen et al., 2005*). The latter bacteria are traditionally called *Haemophilus* spp.

Most Pasteurellaceae from rodents belong to the Rodent cluster which comprises biotype Heyl and Jawetz *P. pneumotropica* and V-factor dependent strains (*Olsen et al., 2005*).

Haemophilus spp frequently occur in contemporary laboratory rat and guinea pig colonies (*Nicklas 1989*; *Nicklas et al., 1993*; *Boot et al., 1994/5*). *Haemophilus* infected rats of various strains usually show hyperaemic bronchial lymph nodes, peribronchiolar hyperplasia and multifocal interstitial pneumonia (*Nicklas 1989*; *Nicklas et al., 1993*; *Boot et al., 1999*).

Haemophilus strain H21 has been found to induce tracheal hyporeactivity to carbachol in mice

and guinea pigs used in asthma research as did *P. pneumotropica* and other growth factor independent species (*Boot et al., 1999*). *Haemophilus* spp may significantly reduce antibody development to *Streptococcus pneumoniae* in rats presumably by interference with the recruitment of neutrophil leucocytes (*Craig et al., 2009*). Importantly *Haemophilus* infection should be absent from rats used for potency testing of *Haemophilus influenzae* type b vaccine.

Infection by Pasteurellaceae including *Haemophilus* species can be detected by culture, polymerase chain reaction (PCR) (*Bootz et al., 1998*; *Boot et al., 2009*) and by serology (*Boot et al., 1994/5*; *Boot et al., 2005*). We use host species specific antigen panels for large scale serological testing of animal colonies and confirm serological observations by culture and/or PCR. Based on cross-reactivity studies (*Boot et al., 1996/7*; *Boot et al., 1999*) the antigen panel for rats includes two *Haemophilus* strains, namely rat strain H21 that is in the Rodent cluster and guinea pig strain H35 that belongs to the *H. parainfluenzae*-complex (*Boot & Reubsaet, 2009*).

In a study using young rats from a *Haemophilus* infected breeding colony we observed a decline in antibody activity against both *Haemophilus* antigens. This observation suggested waning of maternal antibodies which prevent production of antibodies by the young. Young rats will only seroconvert after maternal antibodies have fallen below a threshold (*Ziang & Ertl, 1992*; *Sigrist, 2003*). This implies a transition period with low antibody activity in young rats which might have consequences for the outcome of serological monitoring.

We studied antibody activity to *Haemophilus* antigens in young rats born from *Haemophilus* infected dams by enzyme-linked immunosorbent assay (ELISA) and immunoblot (IB). Young showed high maternal antibody activity until weaning, a decline to a low level between 9 and 12 weeks of age, and then seroconversion. The major consequence of the age-related variation in antibody activity was a low seroprevalence during the transition period and young were temporarily negative by *Haemophilus* H21 ELISA.

Materials and methods

Ethical note

Animal experiments were approved by the institute's Ethical Committee on Animal experiments and were conducted in compliance with national legislation which is based on European Community Directive 86/609/EEC.

Animals

Eight mated female HsdCpb:WU rats aged 6-7 months were obtained from a *Haemophilus* infected breeding colony. They delivered 84 young within a period of one week. Litter size ranged from 5-13. To study antibody activity in individual young rats over time, four rats per litter comprising 2 males and 2 females from 7 litters and 3 males and 1 female from 1 litter were sampled.

Housing

Mothers delivered in a type III macrolon® cage and raised their young until weaning at 3 wks. At weaning four male and four female groups were formed from male (n=17) and female (n=15) young rats from litters 1 and 2; 3 and 4; 5 and 6; and 7 and 8 respectively and housed in open type III cages within the same animal room. The groups contained 3 (one group), 4 (six groups) or 5 (one group) rats. No other laboratory animal species or rat strains were present in the animal room.

Samples

Dams were blood sampled at delivery and at weaning of their young (16 samples).

From each of the 32 young 8 consecutive blood samples were obtained at 3 week intervals from 3 to 24 weeks (256 samples). The remaining 52 littermates were blood sampled when sacrificed at birth (n=8), or at 1 (n=7), 2 (n= 7) or 3 weeks (n= 30) of age.

Samples from rats at birth and at 1 week of age were obtained after decapitation.

All other samples were obtained under KRA [Ketamine (Alfasan, Woerden, The Netherlands) 90 mg/kg intraperitoneally, Rompun (Bayer AG, Leverkusen, Germany) 10 mg kg intraperitoneally, atropine (Vetinex Animal Health, Bladel, The Netherlands) 0.05 mg/kg intraperitoneally] anesthesia through puncture of the orbital plexus or cardiac puncture when rats were sacrificed. Sera were stored at -20 °C until testing.

Pharyngeal samples for culture and PCR analysis were obtained from the dams at weaning of their young and from young sacrificed at 3 (n= 30) and 24 weeks of age (n= 32).

Serology

ELISA

The ELISA was carried out as described previously using whole cell antigens of *Haemophilus* strains

H21 and H35 and various other bacteria (*Boot et al., 1995*). ELISA activity was measured as optical density (OD) minus the OD of the negative control. We included serology using antigen of *Bordetella bronchiseptica* as a representative of absent infections (negative control).

For the preparation of whole cell antigens both *Haemophilus* strains and *B. bronchiseptica* were grown on chocolate agar and plain sheep blood agar respectively for 18 hrs at 37 °C under 7.5% CO_2, harvested in 0.07 M phosphate buffered saline (PBS) pH 7.2, washed 3 times by centrifugation (300 g, 20 min) in PBS and finally resuspended in PBS containing 0.01% (w/v) merthiolate. The antigens were adjusted to 7.5 g/l of protein and coated to polyvinylchloride flat bottomed microtitre plates (Titertek type III, Flow Lab. Inc., Mc Lean, VA, USA).

Sera were tested as 1:50 dilutions for IgG antibodies using horseradish peroxidase conjugated sheep anti-rat IgG (Sigma Aldrich Chemie BV, Veenendaal, The Netherlands) as the secondary antibody. Autologous positive control sera raised by immunisation and negative control sera (from *Pasteurellaceae-* and *B. bronchiseptica*-free rats) were run in each test.

Immunoblot (IB)

In 6 litters young showed a clear age-related variation in ELISA antibody activity.

For these 6 dams serum collected at delivery and serum from one young per litter at 3 weeks ('peak'), 12 weeks ('dip') and 24 weeks ('peak') were tested for antibodies to both *Haemophilus* antigens by IB.

The IB was essentially carried out as described for *Streptobacillus moniliformis* (*Boot et al., 2006*). *Haemophilus* cells were grown, harvested and washed as described above and were boiled (150 mg/l protein) in tap water for 3 min. Proteins were electrophorized on 10% polyacrylamide gels in sodium dodecyl sulfate (SDS). Antigens were electrophoretically transferred onto nitrocellulose membrane (Protran BA83, Schleicher & Schuell, Dassel, Germany). Blots were incubated overnight at 4 °C in 0.05% Tween20/phosphate buffered saline (PBS/Tween) containing 1% low fat milk (Protifar, Nutricia, Zoetermeer, The Netherlands) [and frozen at -20 °C until use]. Immunodetection was performed by incubation of the blots with serum samples diluted 1:50 in PBS/Tween for 60 min. at room temperature. After washing in PBS/Tween, the blot was incubated with peroxidase conjugated sheep anti-rat IgG (Sigma Aldrich Chemie BV, Veenendaal, The Netherlands) 1:2,000 diluted in PBS/Tween. After washing in PBS/

Tween, tetramethyl benzidine/dioctyl sodiumsulfosuccinate (TMB/ DONS 0.06% and 0.2% respectively in dimethyl sulfoxide) was added as substrate. Positive and negative control sera and molecular weight marker with a range 10 to 250 kDa (Kaleidoscope Prestained Standard 161-0375, Biorad Laboratories Ltd, Veenendaal, The Netherlands) were included in each run.

Culture

Pharyngeal samples were cultured for respiratory bacterial infections (notably by *Pasteurellaceae* and *B. bronchiseptica*) on plain sheep blood agar and chocolate agar which were incubated for 24-48 hours at 37°C and read. *Haemophilus* suspected growth was identified and typed by the API NH system.

PCR analysis

Pharyngeal samples were PCR tested for *Pasteurellaceae* infection using the primer set as described by Bootz *et al.* (*Bootz et al., 1998*; *Boot et al., 2009*).

Statistical analysis

Optical density (OD) measured by ELISA with each of the 3 antigens was expressed as a percentage of the activity of the autologous positive control serum [(OD Sample / OD C+) x 100%] or in short: S/C+ %]. In this way day to day variations in ELISA outcome due to time and temperature differences in successive runs were minimized.

For the calculation of seroprevalence(s) S/C+ percentages ≥ 30 were considered positive. Although this percentage may seem an arbitrary cut-off level, our ELISA results generally agree with the outcome of culture and/or PCR for colonies of infected and uninfected rats of various strains with different background (enteric) flora.

The experimental (and statistical) unit is the entity that can be assigned at random to one of the treatments, independently of all other experimental units. Any two experimental units must be able to be assigned to different treatments (*Festing & Altman, 2005*). This implies that in this study the dam is the experimental unit. All statistical analyses were carried out according to Petrie and Watson (*Petrie & Watson, 1995*) using a SPSS® for Windows (version 15.0) computer program (SPSS Inc., IL, USA). Two-sided, exact (i.e. for the non-parametric tests) (*Mundry & Fischer, 1998*) probabilities were estimated throughout. The probability of a Type I error < 0.05 was taken as the criterion of significance.

The antibody activity data were continuous and were summarized as means with standard deviation (SD). The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the data. All results were normally distributed. In addition, percentages of positive test results (seroprevalence) in the colony over time were calculated. To assess the relationship between maternal antibody activity at delivery or after 3 weeks and in offspring at 0 or 3 weeks of age Pearson's linear correlation coefficients (r) were calculated; significance was assessed by a two-tailed test based on the *t* statistic. Differences in ELISA antibody activity between dams at delivery or after 3 weeks and young at 0 or 3 weeks of age, were evaluated with the paired Student's *t* test. Antibody activity data were subjected to an univariate repeated measures ANOVA with a Huynh-Feldt correction and (depending on the comparison) between-subject factors comprising antigen, gender and time. In the analysis type IV sums of squares were used. Homoscedasticity was tested using the Levene's test, which is a powerful and robust test based on the F statistic (*Lim & Lo, 1996*).

IB reactivity to the *Haemophilus* antigens was for all individual sera calculated using the formula [a.1] + [b.2]+ [c.3] in which a, b, c is the number of immunoreactive antigens showing a reaction scored as 1

(light); 2 (medium) or 3 (heavy). For both *Haemophilus* antigens the Spearman's rank correlation coefficient (R_s) was calculated between IB reactivity scores and ELISA reactivity; significance was assessed by a two-tailed test based on the *t* statistic.

Results

Bacteriology

Haemophilus spp infection was detected in all dams, in 23 out of 30 young at weaning and in 29 out of 32 young at the end of the study by culture and in all animals by PCR. *B. bronchiseptica* was not cultured from any animal. *Haemophilus* strains were of a variety of API NH biotypes (data not shown).

Serology

Transmission of maternal antibodies
At delivery (week 0) and after 3 weeks dams showed appreciable ELISA antibody activity to both *Haemophilus* antigens (Table 1) and most young had at 0, 2 and 3 weeks high antibody activity to the H21 antigen and all young had high activity to the H35 antigen. At delivery and after 3 weeks the antibody activity to both *Haemophilus* antigens was significantly higher in sera from the mothers than from

Table 1. ELISA antibody activity to *Haemophilus* strains H21 and H35, and *B. bronchiseptica* antigens in sera from *Haemophilus* infected rat dams and their young.[1]

Antigen	Dams (n = 8)[2] at		Young (n = 8)[2] at age of		
	delivery	after 3 weeks	0 weeks	2 weeks	3 weeks
Haemophilus H21					
Number (percentage) of positive experimental units	6 (75.0)	6 (75.0)	5 (62.5)	6 (85.7)[3]	6 (75.0)
Antibody activity (mean ± SD)	59.4 ± 28.7	70.0 ± 35.4	40.4 ± 25.2	74.9 ± 25.6	46.5 ± 23.3
Dams versus young (i.e. at week 0 of 3)			*P* = **0.000**		*P* = **0.004**
Haemophilus H35					
Number (percentage) of positive experimental units	8 (100)	8 (100)	8 (100.0)	7 (100)[3]	8 (100.0)
Antibody activity (mean ± SD)	117.0 ± 33.6	142.8 ± 15.8	98.0 ± 42.7	138.4 ± 26.7	102.7 ± 25.6
Dams versus young (i.e. week 0 of 3)			*P* = **0.002**		*P* = **0.000**
B. bronchiseptica					
Number (percentage) of positive experimental units	1 (12.5)	1 (14.3)[3]	0 (0.0)	1 (14.3)[3]	1 (12.5)
Antibody activity (mean ± SD)	11.8 ± 12.1	12.4 ± 12.0	6.3 ± 8.1	16.1 ± 13.8	12.6 ± 13.5
Dams versus young (i.e. week 0 of 3)		*P* = 0.057			*P* = 0.589

[1]ELISA = enzyme-linked immunosorbent assay.
[2]Number of experimental units.
[3]Based on 7 experimental units.
[3]*P* values in paired Student's *t* test. Significant differences (*P* < 0.05) are indicated in bold characters. Note that a *P* value of 0.000 does not mean that it is zero, only that it is less than 0.0005.

Table 2. Associations between *Haemophilus* infected rat dams and their young with respect to ELISA antibody activity to *Haemophilus* strains H21 and H35, and *B. bronchiseptica* antigens in serum.[1]

Antigen		Young at age of 0 weeks and dams at delivery	Young at age of 3 weeks dams after 3 weeks
Haemophilus H21			
	Pearson's linear correlation coefficient	*r = 0.956*	*r = 0.933*
	P value	P = **0.000**	P = **0.001**
	Number of experimental units	*n = 8*	*n = 8*
Haemophilus H35			
	Pearson's linear correlation coefficient	*r = 0.984*	*r = 0.823*
	P value	P = **0.000**	P = **0.012**
	Number of experimental units	*n = 8*	*n = 8*
B. bronchiseptica			
	Pearson's linear correlation coefficient	*r = 0.843*	*r = 0.975*
	P value	P = **0.009**	P = **0.000**
	Number of experimental units	*n = 7*	*n = 7*

[1]ELISA = enzyme-linked immunosorbent assay. All associations are significant ($P < 0.05$) and are indicated in bold characters. Note that a P value of 0.000 does not mean that it is zero, only that it is less than 0.0005.

their young. Antibody activity to both antigens in young and their respective mothers was significantly correlated (Table 2).

Neither dams nor their young showed appreciable antibody activity to *B. bronchiseptica* (Table 1).

All litters at birth showed appreciable ELISA antibody activity (i.e ≥ 30% of the positive control serum) to the H35 ($P = 0.003$, paired Student's *t* test, n = 8), but not to the H21 antigen ($P = 0.283$, paired Student's *t* test, n = 8) (Fig. 1, panels a and b). ELISA antibody activity to *B. bronchiseptica* in litters at birth was on average below the threshold ($P = 0.000$, paired Student's *t* test, n = 8, Fig. 1, panel c).

All litters - except litter 3 for which no samples were available at the age of 1 and 2 weeks - showed an increase in antibody activity to *Haemophilus* antigens reaching a peak at 2 weeks and a subsequent decline to a low activity plateau between 9 and 15 weeks.

From 15 weeks all litters showed an increase in antibody activity (seroconversion) to *Haemophilus* antigens and the highest activities were reached at end of the study (24 weeks). As a consequence there was a significant time-effect for both *Haemophilus* antigens in the repeated measurements ANOVA (Table 3). Male and female young of a litter showed a similar pattern of antibody activity which seemed
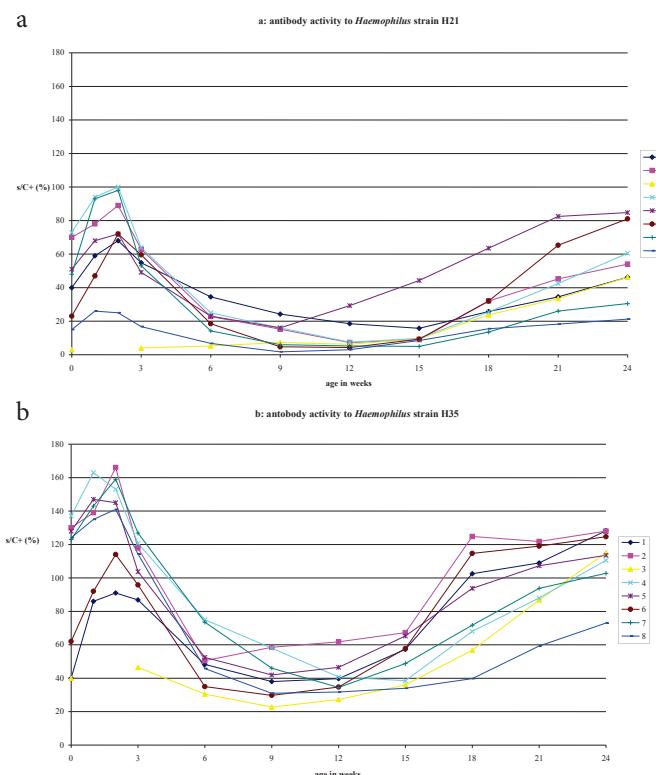
Figure 1. ELISA antibody activity to *Haemophilus* strains H21 and H35 and to *B. bronchiseptica* antigens in litters delivered by 8 *Haemophilus* naturally infected rat dams.
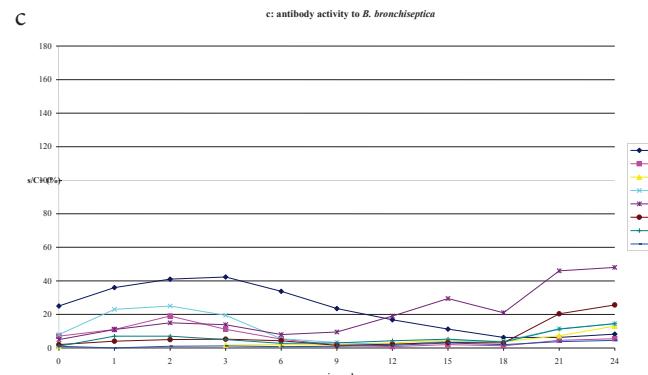
a: antibody activity to *Haemophilus* strain H21

b: antibody activity to *Haemophilus* strain H35

c: antibody activity to *B. bronchiseptica*

Table 3. *P* values in the different repeated measures ANOVA's.[1]

Antigen	Weeks	A[2]	G	T	AxG	AxT	GxT	AxGxT
H21, H35, *B. bronchiseptica*	3, 6, 9, 12, 15, 18, 21, and 24	**0.000**	0.472	**0.000**	0.671	**0.000**	0.592	0.092
H21, H35	3, 6, 9, 12, 15, 18, 21, and 24	**0.000**	0.497	**0.009**	0.560	0.431	**0.008**	0.495
H21	3, 6, 9, 12, 15, 18, 21, and 24	-	0.255	**0.000**	-	-	0.145	-
H35	3, 6, 9, 12, 15, 18, 21, and 24	-	0.824	**0.000**	-	-	0.436	-
B. bronchiseptica	3, 6, 9, 12, 15, 18, 21, and 24	-	0.512	0.171	-	-	0.399	-
H21, H35, *B. bronchiseptica*	0, 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24	**0.000**	-	**0.000**	-	**0.001**	-	-
H21, H35	0, 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24	**0.000**	-	**0.000**	-	0.123	-	-
H21	0, 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24	-	-	**0.000**	-	-	-	-
H35	0, 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24	-	-	**0.001**	-	-	-	-
B. bronchiseptica	0, 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24	-	-	0.232	-	-	-	-

[1]Significant effects (*P* < 0.05) are indicated in bold characters. Note Note that a *P* value of 0.000 does not mean that it is zero, only that it is less than 0.0005.

[2]A = effect of antigen; G = effect of gender; T = effect of time; AxG = interaction effect between antigen and gender; AxT = interaction effect between antigen and time; GxT = interaction effect between gender and time; AxGxT = interaction effect between antigen, gender, and time.

unaffected by the formation of male and female groups by mixing animals from different litters. ELISA antibody activity to *B. bronchiseptica* was in the young between weeks 0 and 24 on average below the threshold.

ELISA activity against the *Haemophilus* H35 antigen was in all samples, and hence in all litters, higher than that to the *Haemophilus* H21 antigen. The ratio of ELISA activity to the H35 and the H21 antigens varied per litter and sampling time.

Antibody activity to *B. bronchiseptica* was at all sampling times at a much lower level than the activity to both *Haemophilus* antigens and only in one litter was a slighty elevated antibody activity to *B. bronchiseptica* found at 2 weeks of age (Fig. 1c).

Seroprevalence
Average antibody levels and percentages of positive test results (seroprevalence), for rats from all litters, to the two *Haemophilus* antigens at each time point is shown in Fig. 2. For both *Haemophilus* antigens there was an age related variation in antibody levels and hence seroprevalence. The variation was most striking for the *Haemophilus* H21 ELISA with a dip between 6 and 15 weeks of age and a 0% seroprevalence at 9 weeks. The seroprevalence measured by the *Haemophilus* H35 ELISA showed a dip at 9-12 weeks but seroprevalence did not fall below 60%.

Immunoblot
As shown in Fig. 1a-b, 6 litters young showed a clear age-related variation in ELISA antibody activity. Sera from the 6 corresponding dams detected various immunodominant antigens in both *Haemophilus* strains and banding patterns differed between dams. The strain H21 antigens most frequently detected measured 10-15, 37, 44 and 100 kDa; the H35 antigens most frequently detected were of 10-15, 40, 57 and 100 kDa size. The banding pattern detected by sera from dams and their young at 2 weeks of age appeared similar.
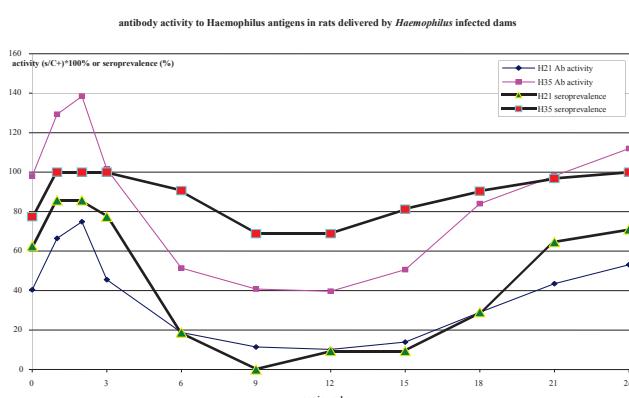

Figure 2. Variation in antibody activity to *Haemophilus* antigens in rats delivered by *Haemophilus* infected dams, leads to variation in seroprevalence

Table 4. Immunoblot reactivity against antigens of *Haemophilus* strains H21 and H35 in naturally *Haemophilus* infected rat dams and their young.

Haemophilus antigen	Dams at birth	Young		
		2 wks	12 wks	24 wks
strain H21	29*	21	5	26
strain H35	22	14	2	18

* Reactivity calculated from the number of immunoreactive antigens and the intensity of the reaction detected by 6 sera per group

Total IB reactivity found in sera from young at 2 weeks of age (Table 4) diminished to a low level at 9-12 weeks (ELISA dip) but IB reactivity reached maternal levels in sera taken from young rats at the end of the study. IB reactivity scores for both *Haemophilus* antigens were significantly correlated with ELISA reactivity (*Haemophilus* H21: R_s = 0.717, P = 0.000081; *Haemophilus* H35: R_s = 0.696, P = 0.000157).

Discussion

Transmission of maternal immunity in rat

The transfer of maternal antibodies from rat dams to offspring occurs partly *in utero* but mainly postnatally via the mammary gland in the first two weeks of life (*Brambell, 1970*; *Bainter, 2007*). We found anti-*Haemophilus* antibody activity immediately after birth (Fig. 1a-b) in offspring delivered by *Haemophilus* infected dams and a rapid increase in antibody activity in littermates in the first two weeks of life (Table 1 and Fig. 1a-b). Antibody activity in young at 0 and 3 weeks of age significantly correlated with levels measured in the dams at delivery and 3 weeks later (Table 2); this agrees with findings in various other species of animal (*Grindstaff et al., 2003*).

ELISA activity measured by the *B. bronchiseptica* antigen represented absent infections in our study. Usually some background ELISA activity is measured in uninfected animals due to cross-reactivity with non-pathogenic bacteria entering the body e.g. via the food. This aspecific activity (Fig. 1c) is also transmitted to the offspring but it is at a much lower level than activity against infecting bacteria such as *Haemophilus* spp (Fig. 1 a, b).

Diminishing maternal antibody in young rats and seroconversion

From 2 weeks of age anti-*Haemophilus* antibody activity declined to a plateau that was reached at about 6-9 weeks and from 12-15 weeks returned to higher levels (Fig. 1 a-b). A similar pattern of decline and seroconversion was found in young delivered by *Corynebacterium kutscheri* infected Wistar-Lewis, Brown Norway (BN) and Fisher rats (*Suzuki et al., 1988*) and by *Mycoplasma pulmonis infected* LEW rats (*Cassell et al., 1986*).

The *M. pulmonis* infected rats seroconverted within 2-4 months whereas the *C. kutscheri* infected rats remained seronegative for about 6 months. The long delay to measurable seroconversion to *C. kutscheri* is likely due to the fact that antibodies to the bacterium were measured by an agglutination assay (*Suzuki et al., 1988*) which is less sensitive than ELISA used to measure activity against *M. pulmonis* or *Haemophilus* antigens (*Cassell et al., 1988*).

At the end of our study (24 weeks) young showed ELISA antibody activities similar to those found in their dams, which is not surprising as dams were 6-7 months of age when they delivered their young.

Immunoblot

That IB reactivity (Table 4) followed variation in ELISA activity and that activities measured by both assays were found to correlate was to be expected. *Pasteurellaceae* including *Haemophilus* spp are Gram negative bacteria with a complex antigenic 'make up' comprising lipopolysaccharides and proteins (*Fenwick, 1995*). Rodents infected by *P. pneumotropica* raise ELISA antibodies against all these kinds of antigen (*Manning et al., 1989*) of which those to immunodominant proteins can be measured by IB.

Age-related IB reactivity has also been found in natural *Pneumocystis carinii* infection in rats which showed antibodies to four major antigens up to 4 weeks, but not at 8 weeks, whereas later IB antibody activity returned as did ELISA activity (*Hong et al, 1995*). IB is used as a confirmatory test for ELISA results for instance in monitoring infection by *C. piliforme* (*Motzel et al., 1991*), *M. pulmonis* (*Simecka & Cassell, 1987*) and viral infections. Age of the animals should be considered in the interpretation of IB test results.

By ELISA the serological behaviour of the young was similar within a litter and apparently not affected by the formation of male and female groups at weaning, whereas differences were seen between the litters (Fig 1 a-b). Similar findings were reported for ELISA reactivity in *M. pulmonis* naturally infected rat litters (*Cassell et al., 1988*). This supports observations that the antibody response to both *M. pulmonis* (*Simecka & Cassell, 1987*) and *Haemophilus* infection (*Boot et al., 2005*) is in rats at least partly under genetic control.

Litter and strain related differences in IB reactivity complicate the interpretation of IB test results.

Seroprevalence and consequences for health monitoring

An important implication of the age-related variation in ELISA antibody levels is an age- related variation in seroprevalence measured for the group (or colony) (Fig. 2).

The prevalence measured by a given assay determines the sample size needed to detect at least one positive animal in a sample (Jonas, 1976). FELASA recommends a sample size of 10 animals (or samples) for the periodic serological health monitoring of rodent colonies which means that infections with a prevalence under 30% will not be detected (*Nicklas et al., 2002*).

The seroprevalence measured by our *Haemophilus* H21 ELISA was under 30% in rats aged 6 to 18 weeks and so the assay, if used alone at these times, would have yielded a negative health monitoring record for the colony. The seroprevalence measured by the H35 ELISA remained above 60% (Fig. 2).

Selection of animals for health monitoring by serology

Our findings lead us to consider that the selection of animals might be adapted to different situations.

- To evaluate a colony of unknown infection history one should test samples from different age categories: around weaning, 9-12 weeks and > 24 weeks (6 months). Males and females seem to be equally suitable for testing but it is advisable to sample animals from different litters. An age-related antibody activity pattern as in Fig. 2 indicates enzootic infection. Antibody activity on a low level as measured to *B. bronchiseptica* in rats < 6 weeks and > 15 weeks (Fig. 1c) suggests aspecific background activity. Antibody activity at the age of 9-12 weeks clearly exceeding activity in other age groups indicates recent infection. Posi-

tive and unequivocal serological findings should be confirmed by culture and/or PCR. If infection cannot be detected by other means the colony may be considered uninfected.

- Subsequent testing of such an uninfected rat colony might be limited to animals aged 9-12 weeks as they will likely seroconvert upon recent infection. In rats exposed to various different pathogenic bacteria in a model mimicking natural infection seroconversion was found from weaning (*Boot, 2001*).

- Rederivation of a rat colony to (re)establish SPF status can involve hysterectomy or embryo transfer. Hysterectomy derived rats foster nursed to SPF dams should be tested (together with sera from donor animals obtained at hysterectomy) at weaning, 9-12 weeks and for instance at 15-18 weeks. Prenatally transmitted maternal antibodies will decline both in infected and in uninfected young. An increase in antibody activity from 15 weeks indicates active infection and hence failure of the rederivation efforts. Difficulties in the interpretation of serological findings can be circumvented by rederiving colonies by embryo transfer to seronegative fosters.

It is obvious that quantification of antibody activity will be helpful in the interpretation of serological findings. We expressed ELISA antibody activity of our samples as a percentage of the activity of the positive control sample. In assays such as IFA (indirect fluorescent antibody) and agglutination titres should be determined

We showed that *Haemophilus* enzootically infected rats have an age-related variation in antibody activity to the infection. Such a variation leads to an age related variation in seroprevalence. This phenomenon will also occur with other enzootic infections in rats. Temporary low antibody activity due to the decline of maternal antibodies is also to be expected in mice as they resemble rats in the transmission of maternal immunoglobulins (*Brambell, 1970*).

We conclude that the likelihood of detection of infections by serology in an enzootically infected colony depends on age of the animals tested.

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