Microbiological monitoring of laboratory mice and biocontainment in individually ventilated cages (IVCs): a field study

Short title: Microbiological monitoring of laboratory mice in IVCs

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23 **Summary**

24 Over recent years, the use of individually ventilated cage (IVC) rack systems in laboratory
25 rodent facilities has increased. Since every cage in an IVC rack may be assumed to be a
26 separate microbiological unit, comprehensive microbiological monitoring of animals kept in
27 IVCs has become a challenging task, which may be addressed by the appropriate use of
28 sentinel mice. Traditionally, these sentinels have been exposed to soiled bedding but more
29 recently, the concept of exposure to exhaust air has been considered. The work reported here
30 was aimed firstly at testing the efficiency of a sentinel-based microbiological monitoring
31 programme under field conditions in a quarantine unit and in a multi-user unit with frequent
32 imports of mouse colonies from various sources. Secondly, it was aimed at determining
33 biocontainment of naturally infected mice kept in an IVC rack which included breeding of the
34 mice. Sentinels were exposed both to soiled bedding and to exhaust air. The mice which were
35 used in the study carried prevalent infectious agents encountered in research animal facilities
36 including mouse hepatitis virus (MHV), mouse parvovirus (MPV), intestinal flagellates and
37 pinworms. Our data indicate that the sentinel-based health monitoring programme allowed
38 rapid detection of MHV, intestinal flagellates and pinworms investigated by a combination of
39 soiled bedding and exhaust air exposure. MHV was also detected by exposure to exhaust air
40 only. The IVC rack used in this study provided biocontainment when infected mice were kept
41 together with non-infected mice in separate cages in the same IVC rack.

42

43 **Keywords**

44 IVC, health monitoring, sentinels, biocontainment, exhaust air sampling

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47 Standardization of husbandry and health parameters in animal experimentation is a
48 prerequisite for *in-vivo* biomedical research. Reproducible results may depend on the use of

49 animals of uniform high microbiological quality (Baker 1998.; Bhatt *et al.* 1986; Hansen
50 1994; Lussier 1988). As such, regular monitoring of laboratory mice and rats has been
51 recommended to obtain information on the health status of experimental and breeding
52 colonies (Nicklas *et al.* 2002). Over recent years, the use of individually ventilated cage (IVC)
53 rack systems in laboratory rodent facilities has increased. In a typical IVC rack, each cage
54 receives high efficiency particle absorbance (HEPA)-filtered air which, when supplied under
55 positive pressure, protects the animals in the cages from airborne infectious or other noxious
56 particulate agents present in the environment (Clough *et al.* 1995; Cunliffe-Beamer and Les
57 1983; Lipman 1999; Lipman *et al.* 1993). Similarly, the exhaust air from the cages is
58 normally also HEPA-filtered before it is returned into the room environment. Thus, transfer of
59 infectious agents from cage to cage within a given IVC rack or room is minimised (Gordon *et*
60 *al.* 2001; Lipman 1999). These characteristics have significantly contributed to maintaining
61 the health status of colonies, particularly when animals with different microbiological status
62 have been held in close vicinity (Josten *et al.* 1999).

63 Since every cage in an IVC rack may be assumed to provide its own zone of
64 biocontainment, comprehensive microbiological monitoring within the rack has become a
65 challenging task. Random sampling of research animals in each room or a sample from cages
66 in an IVC rack requires the use of potentially valuable animals and is normally not acceptable
67 to investigators. Therefore, the use of sentinels for monitoring of mice kept in IVC racks has
68 become the method of choice. To avoid interference with the breeding programs or
69 experiments, as is the case with contact sentinels, the use of sentinels exposed to soiled
70 bedding has been developed (Nicklas *et al.* 2002; Thigpen *et al.* 1989; Wilhelm *et al.* 2002).
71 However, soiled bedding sentinels may not pick up airborne agents such as Sendai virus or
72 cilia associated respiratory (CAR) bacillus which are generally not transmitted by the faecal-
73 oral route(Artwohl *et al.* 1994; Cundiff *et al.* 1995; Dillehay *et al.* 1990).

74 To alleviate this problem, IVC racks which provide the sentinels with exhaust air from
75 the entire IVC rack were described. In addition, it was proposed to locate particle filters in the
76 exhaust air to track airborne infectious agents (Schmidt and Brielmeier 2001). The efficacy of
77 these developments has been tested in detail using mice experimentally infected with MHV,
78 MPV, mouse rotavirus (Epizootic Diarrhoea of Infant Mice, EDIM), Sendai virus and
79 *Helicobacter* spp. (Compton *et al.* 2004b). Although this experiment provided detailed insight
80 into the efficacy of different sentinel monitoring approaches, it may not reflect the situation
81 under field conditions where mice are undergoing different stages of the infectious cycle at
82 any one time or where chronic or persistent infections may be a significant issue. In the
83 present work, the effectiveness of sentinel monitoring of naturally infected mice and
84 biocontainment in an IVC rack was determined under field conditions.

85 The mice which were monitored carried prevalent infectious agents in research animal
86 facilities including MHV, MPV, intestinal flagellates (*Enteromonas* spp, *Trichomonas* spp,
87 *Chilomastix* spp) and pinworms (*Syphacia obvelata*). These infectious agents differ in size,
88 infectivity, mode of transmission and stability in the environment. MHV is a highly
89 contagious enveloped RNA coronavirus (80-160 nm). Several MHV strains with tropism for
90 different tissues exist. In a natural infection, enterotropic MHV is restricted largely to the
91 intestine with excretion primarily in faeces while respiratory MHV is disseminated from the
92 nasal mucosa to various target organs (Barthold *et al.* 1993). Infections are usually self-
93 limiting in the absence of breeding. MHV is transmitted by direct contact, the faecal-oral
94 route, aerosols or fomites (Compton *et al.* 1993). Like other enveloped viruses, MHV is
95 relatively unstable in the environment. MPV is a non-enveloped single stranded DNA virus
96 (18-25nm) and is moderately contagious. Mice are infected primarily by direct contact with
97 virus shed in faeces or urine. Infections are usually chronic with extended duration of virus
98 shedding (Jacoby *et al.* 1996). Like all parvoviruses, MPV is capable of surviving in the
99 environment for weeks which makes fomite transmission more likely. In mice, *Trichomonas*

100 spp., *Chilomastix* spp. and *Enteromonas* spp. live in the caecum. The main route of infection
101 of these protozoans is via ingestion of the trophozoite (*Trichomonas* spp., *Chilomastix* spp.,
102 *Enteromonas* spp.) or the cysts (*Chilomastix* spp., *Enteromonas* spp.) which are passed in the
103 faeces of infected animals (Flynn 1973). These intestinal flagellates are not considered as
104 pathogens. The infection is persistent in an infected mouse colony without the appearance of
105 clinical signs. *Syphacia obvelata* is a caecal pinworm with a direct life cycle of 11 to 15 days.
106 It deposits its eggs (120 x 35 µm) in the peri-anal region of the mice. Infection occurs via
107 ingestion of the eggs. The eggs of *S. obvelata* have been shown to aerosolize, which makes
108 transmission via the air likely.
109 Our studies were carried out in IVC racks under field conditions in two different
110 breeding and holding areas: a) in a quarantine unit, designated Q-study, with restricted user
111 access. In this study, the use of sentinels exposed to soiled bedding or to exhaust air of the
112 IVC rack or to a combination of both was compared; b) in the German Mouse Clinic
113 (Brielmeier *et al.* 2002; Gailus-Durner *et al.* 2005), designated GMC-study. In this study, the
114 efficiency of a specific sentinel programme tailored to monitor a multi-user unit with frequent
115 imports of mouse colonies from numerous sources of variable health status was determined.
116 In both studies, the sentinel-based health monitoring programme allowed rapid detection of
117 infectious agents investigated by exposure to both soiled bedding and exhaust air. Moreover
118 the IVC racks provided biocontainment when infected mice were kept together with non-
119 infected mice in separate cages in the same IVC rack.
120
121
122 **Material and methods**
123
124 **Mice** Naturally infected immunocompetent inbred mice, obtained from different breeding
125 and experimental animal facilities, together with their progeny were used as carriers of

126 infectious agents and hereafter named index mice (IM). All IM had a continuous record of
127 regular microbiological monitoring according to the FELASA Recommendations (Nicklas *et*
128 *al.* 2002) prior to importation into the quarantine unit or the GMC. Outbred Swiss (CD-1)
129 mice, 6-8 weeks old, were used as sentinels, as negative control mice and as breeding
130 partners. They were obtained from the GSF full barrier breeding unit which had been
131 routinely monitored at 6-week intervals to a higher standard than the FELASA
132 Recommendations (Nicklas *et al.* 2002). This breeding unit was examined every 6 weeks. The
133 serological examinations performed were to the annual standard (Nicklas *et al.* 2002) with the
134 addition of Leptospira serogroups, ballum, canicola, hebdomadis and icterohaemorrhagiae, K
135 virus, Lactate dehydrogenase virus, Polyoma virus, Mouse thymic virus and Hantaviruses
136 (Kraft *et al.* 1994). The mice were found consistently negative for all of the FELASA-listed
137 infectious agents including the ones examined in this study.
138
139 **Mouse husbandry and cage changes** Mice were kept on wood shavings (Altromin, Lage,
140 Germany) in type II Makrolon cages in double-sided IVC racks (VentiRacks™; BioZone,
141 Margate, UK), each holding 84 cages. Each IVC rack was fitted with 1 (GMC-study) or 2 (Q-
142 study) BioScreen™ sentinel cages where sentinels received a proportion of the total exhaust
143 air of the IVC rack at 1.0 Pa positive air pressure. The technical performance, including the
144 air change rate of the IVC racks, was continuously monitored by a DigiFlow™ system
145 supplied by the manufacturer. The mice were fed a standard mouse diet (1314 Altromin,
146 Lage, Germany) and offered autoclaved water *ad libitum* in bottles. Room conditions were set
147 to 22 to 24°C, relative humidity of 50-60 % and a 12/12 hour light/dark cycle. The Q-study
148 was carried out in a quarantine unit with the rooms at negative differential pressure to the
149 corridor. The GMC-study was carried out in an eleven-module unit with the room at positive
150 differential pressure to the corridor (Fig. 1). The IVC racks operated in their standard mode of
151 120 air changes per hour with 1.5 Pa positive cage pressure relative to the holding room.

152 To simulate field conditions, animal care staff were unaware of the infectious status of
153 the mice in the IVC racks. Before entering a mouse room, staff were clothed in a clean suit
154 and gown and wore disposable gloves, hats and face masks. Cages were changed weekly in
155 the order shown in Fig. 2 as is normal practice in the two units. During routine changes of
156 cages including lids, wire bars and water bottles in Class II laminar flow changing stations,
157 mice were transferred to new cages with forceps padded with silicone tubing. Forceps were
158 disinfected after each cage change with 70 % ethanol. All materials, including IVC racks,
159 cages, lids, feeders, bottles, bedding and water were autoclaved before use. Aliquots of
160 approximately five cm^3 of bedding were taken from each used cage of index and control mice
161 on a rack. These aliquots were mixed in a sterile box with an equivalent amount of new sterile
162 bedding, and the resultant mixture, hereafter called soiled bedding, was distributed to the
163 sentinel cages of the same rack.
164
165 **Microbiological examination** At the time of examination, the mice were delivered live in
166 filter-topped boxes from the IVC rack to the necropsy room at the GSF and euthanised with
167 0.2 mL of anaesthetic intraperitoneally (thiopental-sodium (33 mg/mL) dissolved in 0.9%
168 sodium chloride). Collection of samples was performed using full aseptic techniques (Kraft *et*
169 *al.* 1994; Needham 2000; Needham 1979). The procedures relevant for detection of the
170 infectious agents are briefly described as follows. Blood was collected in serum gel tubes
171 (Vetlab Supplies, Sussex, UK) from the thorax after opening the vena cava and the heart and
172 thoroughly mixed. After standing at room temperature for 45 minutes, serum was prepared by
173 centrifugation at 5000g for 10 min. The serological tests for MHV and MPV were performed
174 using ELISA following inactivation of the serum samples at 56 °C for 45 min immediately
175 prior to testing. Caecal contents were expressed into sterile petri dishes for inspection under
176 low power microscopy (12x and 20x magnification) for the presence of helminths. Wet
177 preparations of the caecal contents were made with sterile phosphate buffered saline and

178 examined for intestinal protozoa at 40x magnification using phase contrast microscopy. The
179 flagellates were differentiated according to the morphology of their trophozoites (Flynn
180 1973). Cellophane tape impressions of the anus, skin and fur were collected (Flynn 1973) and
181 evaluated using a stereo microscope at 20x magnification. All microbiological examinations
182 with the exception of the Syphacia diagnosis in the GMC study were performed by The
183 Microbiology Laboratories, North Harrow, Middlesex, England.
184
185 **Q-Study: Experimental groups** The Q-study was performed as two consecutive
186 experiments, Q-1 and Q-2, each carried out for three months. In both experiments, 4-12 week-
187 old mice from different breeding and experimental animal facilities were used as carriers of
188 infectious agents and kept in an IVC rack. In experiment Q-1, a total of 95 mice were
189 obtained from 2 non-barrier breeding and experimental facilities in which health monitoring
190 with soiled bedding sentinels 6 to 12 weeks prior to commencement of the experiment had
191 revealed the presence of MHV, *Enteromonas* spp, *Trichomonas* spp, and *Chilomastix* spp. To
192 simulate breeding under field conditions, a total of 42 breeding pairs were allocated and 11
193 mice were kept singly. From the offspring born during the three-month experimental period,
194 61 were kept in 16 cages until the end of the experiment. The remaining offspring were
195 excluded from the experiment at weaning. In total, 156 IM were used as potential carriers of
196 infectious agents.
197 In experiment Q-2, 98 mice were derived from five non-barrier breeding and
198 experimental facilities in which FELASA-conforming health monitoring with soiled bedding
199 sentinels 6 to 12 weeks prior to commencement of the experiment revealed the presence of
200 MHV, MPV, *Enteromonas* spp, *Trichomonas* spp and *Chilomastix* spp. Eight additional
201 females, seronegative for antibodies to the viruses and free of the flagellates investigated,
202 were included as contact animals and for matings. From these 106 mice, 20 breeding pairs
203 including 8 additional females were allocated and 66 mice were kept singly. From the

204 offspring born during the three-month experimental period, 91 were kept in 24 cages until the
205 end of the experiment, the remaining offspring were excluded from the experiment at
206 weaning. In total, 197 IM were used.
207
208 **Q-Study: Sentinel monitoring and biocontainment** To detect the agents carried by the IM
209 in the IVC rack, a sentinel-based health monitoring programme was implemented (Nicklas *et*
210 *al.* 2002). In Experiment Q-1, four soiled bedding sentinels (SBS) were kept in each of 2
211 cages on soiled bedding (Fig 2). Further four combined sentinels (CS) were kept in each of
212 two BioScreen^{TM} cages on soiled bedding and received as supply air a proportion of the total
213 exhaust air from all cages. At day 42 and at day 84, two SBS and CS from each cage were
214 taken for examination.
215 In Experiment Q-2, two and four soiled bedding sentinels (SBS) were kept in each of 2
216 cages on soiled bedding (Fig 2). Two and four exhaust air sentinels (EAS) were kept on
217 sterile bedding in each of two BioScreen^{TM} cages and received as supply air, a proportion of
218 the total exhaust air from all cages. At day 42, two SBS and EAS from the cages with the two
219 sentinels were taken for examination and replaced by two new sentinels for the monitoring
220 period days 43 to 84. At this time point, two SBS and EAS from the cages with four sentinels
221 were taken for examination. The remaining two were examined at day 84 for the monitoring
222 period day 1 to 84. Each sentinel cage contained approximately 800 cm^3 of soiled bedding.
223 Soiled bedding was renewed weekly.
224 Eight cages with four negative controls per cage were distributed randomly in the IVC
225 rack as shown in Fig. 2. These mice were used to investigate biocontainment of the IVCs,
226 defined as absence of cage–to-cage infection, and dissemination of infectious agents during
227 husbandry procedures during the Q-study. Cage changes were carried out in the sequence
228 shown in Fig. 2. In both experiments Q-1 and Q-2, two mice from each cage were examined
229 on days 42 and 84, respectively.

230

231 **Q-Study: Detection of infectious agents** On days 42 and 84 after commencement of
232 Experiment Q-1, control mice and sentinels were subjected to complete necropsy with
233 serological, bacteriological and parasitological investigations as described above. In addition,
234 randomly selected progeny or parent IM (one mouse, at least 4 weeks of age, every 3rd cage of
235 the rack) were investigated on day 84, i.e. at the end of the study, to confirm their
236 microbiological status.

237 Control mice and sentinels were investigated over 4 monitoring periods: days 1-28
238 (only serology for MHV and MPV), days 1-42, days 43-84 and days 1-84. Two to four
239 sentinels and 16 controls (two from each cage with four mice) were investigated at days 42
240 and 84 by complete necropsy with serological, bacteriological and parasitological
241 investigations.

242

243 **GMC-Study: Workflow** In the GMC, colonies comprising 60-70 test mice of a mutant
244 strain are frequently imported. Upon arrival, mice are transferred from transport boxes into
245 autoclaved type II cages in class II laminar air flow changing stations (Fig. 1, F, Ehret,
246 Emmendingen, Germany), brought through a sluice (Fig. 1, G) into the GMC, placed into
247 newly autoclaved IVCs (Fig. 1, C) in the mouse room (Fig. 1, B) of the first module (Fig. 1,
248 A) and subjected to phenotyping analysis. Thereafter, they are transferred to the other 10
249 modules in a particular sequence according to the phenotyping workflow, where they remain
250 for varying periods of time.

251

252 **GMC-Study: Sentinel monitoring and biocontainment** In the GMC-study, combined
253 sentinels were used as described above. Each module contained two to three IVC racks. In
254 order to increase the frequency of examinations in these modules, sentinels in alternate IVC
255 racks were investigated at 6-weeks intervals. Therefore, the monitoring period was three

256 months for each of the two to three IVC racks in each module but 6 weeks for each module.
257 Whereas new sentinels were placed in the BioScreen™ sentinel cages at three-month
258 intervals, the colonies of test mice remained in the IVC racks of a given module for the length
259 of time required for phenotyping. Biocontainment defined as absence of cage–to-cage, IVC
260 rack-to-IVC rack or module-to-module transmission of *S. obvelata* was investigated by
261 continuous sentinel monitoring of all IVC racks of the GMC and additional repeated analyses
262 of anal tapes taken from the two suspected colonies over 7 months.
263
264
265 **Animal welfare** The procedures reported here were not considered as animal experiments
266 under German Law and therefore not subject to formal ethical review. However, the
267 husbandry of the animals and all animal procedures were in accordance with the Animal Care
268 and Use regulations of the GSF and with German Legislation.
269
270
271 **Results**
272
273 **Experiment Q-1: Microbiological monitoring using soiled bedding and combined**
274 **sentinels** In experiment Q-1, the efficiency of microbiological monitoring using soiled
275 bedding sentinels (SBS) was compared with that of using combined sentinels (CS). The
276 position of the cages with the different types of mice on the IVC rack is shown in Fig. 2a and
277 2b. The results of experiment Q-1 are summarized in Table 1.
278
279 **Experiment Q-1: Microbiological status of Index mice** On day 84, three out of 20
280 randomly taken index mice, were seropositive for MHV. *Enteromonas* spp and *Chilomastix*
281 spp were detected in 11 and 8 mice, respectively. *Trichomonas* spp (0/20) were not detected.

Experiment Q-1: Sentinel monitoring On day 42 and day 84 of the study, SBS and CS were investigated. On day 42, antibodies to MHV were detected in SBS (4/4) and in CS (3/4). *Enteromonas* spp was detected in both SBS (2/4) and CS (2/4). *Trichomonas* spp (0/4) and *Chilomastix* spp (0/4) were not detected in any of the sentinels. On day 84, antibodies to MHV were detected in SBS (3/4) and CS (4/4). *Enteromonas* spp (0/4), *Trichomonas* spp (0/4) and *Chilomastix* spp (0/4) were not detected. No other FELASA-listed bacteria or viruses were detected in the sentinels.

Experiment Q-1: Biocontainment/Control mice Two negative control mice from each of the eight cages, necropsied on days 42 and 84, respectively, were negative for antibodies to MHV (0/16) and for *Enteromonas* spp (0/16), *Trichomonas* spp (0/16) and *Chilomastix* spp (0/16). In addition, FELASA-listed bacteria or viruses were not detected in the control mice.

Experiment Q-2: Microbiological monitoring using soiled bedding and exhaust air sentinels In experiment Q-2, the efficiency of microbiological monitoring using SBS was compared with that of exhaust air sentinels (EAS). Furthermore, four different monitoring periods, day 1-28, day 1-42, day 43-84 and day 1-84, were investigated to determine the time point of detection of the infectious agents. The cage loading of the IVC rack is shown in Fig. 2c and 2d. The data of experiment Q-2 are summarized in Table 2.

Experiment Q-2: Microbiological status of index mice On day 84, index mice were seropositive for MHV (12/36) and MPV (3/39). *Enteromonas* spp (4/43), *Trichomonas* spp (1/43) and *Chilomastix* spp (2/43) were also detected.

308 **Experiment Q-2: Sentinel monitoring** On day 28, antibodies to MHV were detected in
309 SBS (1/5) and EAS (2/5). On day 42, antibodies to MHV were detected in SBS (4/4) and EAS
310 (4/4). Flagellates were not detected. On day 84, (monitoring period days 43 to 84), antibodies
311 to MHV were detected in SBS (2/2) but not in EAS (0/2). Flagellates were not detected. On
312 day 84 (monitoring period days 1 to 84), antibodies to MHV were detected in both SBS (2/2)
313 and EAS (2/2). *Enteromonas* spp, *Trichomonas* spp and *Chilomastix* spp were each detected
314 in SBS (1/2) but not in EAS (0/2). MPV antibodies were not detected during the experiment.
315 Furthermore, other FELASA-listed bacteria and viruses were not detected.
316
317 **Experiment Q-2: Biocontainment** On day 28, sera from three control mice taken randomly
318 from three different control cages were negative for antibodies to both MHV (0/3) and MPV
319 (0/3). On day 42 and on day 84, 16 control mice, 2 each from the 8 control cages (Fig. 2c and
320 2d) were negative for antibodies to MHV (0/16), MPV (0/16) and for *Enteromonas* spp
321 (0/16), *Trichomonas* spp (0/16) and *Chilomastix* spp (0/16). Furthermore, other FELASA-
322 listed bacteria and viruses were not detected in the control mice.
323
324 **GMC-Study: Monitoring of *S. obvelata*** Phenotyping in the GMC requires a constant
325 workflow of colonies of test mice with various periods of stay in the eleven different modules
326 during the primary screen. Colonies are normally split into two groups that are transferred
327 between the modules of the GMC independent of each other in different sequences. Thus, at
328 one time point, a colony of mice is kept in two different modules. During the experimental
329 period, 60 mice of a colony of originally 75 immunocompetent mice were kept in 13 cages in
330 modules A, B, C, D and E in IVC racks A2, B2, C3, D1 and E1 for 106, 106, 53, 68 and 53
331 days, respectively, depending on the time needed for phenotyping. During regular
332 microbiological monitoring at three-month intervals *S. obvelata* was detected in sentinels of
333 IVC racks A2 and B2. Sentinels from IVC racks C3, D1 and E1 were not examined at that

334 time point. Continuous detailed records of workflow in the GMC lead to the identification of
335 2 colonies suspected to be infected. These had been imported from 2 different external mouse
336 facilities 6 weeks earlier. Eleven days after detection of *S. obvelata*, all mice in these two
337 colonies and all sentinels in the GMC were examined by anal tape and the infection was
338 attributed to 1 of the 2 suspected colonies. After a further 7 days, all mice of the infected
339 colony were euthanized by CO_2 inhalation, anal tapes were taken and the gut contents were
340 examined. In total, 22 out of 60 mice were positive; whereas eggs were detected in 17 mice
341 worms were detected in the gut contents of 20. From the 17 mice tested positive by anal tape,
342 15 were positive when the gut contents were examined. From the 20 tested positive by
343 examination of the caecal contents, 15 were positive when examined by anal tape. The results
344 are summarised in Table 3.
345
346 **GMC-Study: Dynamics of *S. obvelata* detection in IVC racks** In the GMC, the mice were
347 kept in IVC racks equipped with BioScreen™ cages in which the combined sentinels were
348 exposed to used bedding and exhaust air. During the period of stay of the infected colony
349 mentioned above, the sentinels in the BioScreen™ cages of IVC racks A2, B2, C3, D1 and E1
350 were exposed to bedding of the infected colony for 7, 27, 13, 5 and 14 days, respectively.
351 Since transfer of colonies between GMC modules is independent of the cage changes carried
352 out at fixed intervals (e.g. every Monday), exposure to exhaust air from upstream cages was
353 11, 28, 14, 10 and 18 days, respectively. From the date of first contact to soiled bedding to the
354 date of subsequent *S. obvelata* diagnosis either by the regular three-month or weekly anal tape
355 examination the time periods between contact and diagnosis were calculated as 38, 36, 27, 27
356 and 14 days, respectively. The same was calculated for the time period between first contact
357 to exhaust air and subsequent diagnosis as 42, 37, 28, 32 and 18 days, respectively. In
358 summary, exposure of combined sentinels to soiled bedding for at least five days and exhaust
359 air for at least 10 days was sufficient to transmit *S. obvelata* from the infected colony to the

360 sentinels in IVC rack D1. The shortest interval from exposure of combined sentinels to soiled
361 bedding and exhaust air to detection of *S. obvelata* was 14 and 18 days, respectively, as
362 observed for IVC rack E1. The data of the GMC study are summarised in Table 4.
363
364 **GMC-Study: Eradication, biocontainment and follow-up** Eighteen days after detection of
365 *S. obvelata,* the infected colony was culled by exposure to carbon dioxide. All remaining mice
366 from IVC racks B2, C3 and D1 including their sentinels were placed in freshly autoclaved
367 IVC racks. The five potentially contaminated IVC racks A2, B2, C3, D1 and E1 were
368 autoclaved. Phenotyping equipment and laboratory bench tops in the infected modules were
369 disinfected on five days per week for six weeks (PurseptA, Merz, Frankfurt, Germany). The
370 floors of the mouse rooms of the five affected modules were wet-cleaned with 1.5% Pursept
371 FD (Merz, Frankfurt, Germany). The floors of the laboratories were wet-cleaned twice
372 weekly. Anal tape examination at 7-day intervals for 6 subsequent weeks revealed the
373 presence of *S. obvelata* in the sentinels of the IVC racks B2, C3 and D1. These sentinels were
374 euthanized and replaced. *S. obvelata* was not detected in the new sentinels of the five IVC
375 racks during seven subsequent months covering two rounds of monitoring in three-month
376 intervals. During this time, 11 colonies comprising 412 mice were kept in these five IVC
377 racks. Moreover, *S. obvelata* was not detected during this time in any sentinels, which
378 monitored the 3.800 mice kept in the remaining modules of the GMC.
379

380 **Discussion**

381 Although the use of IVC rack systems in laboratory rodent facilities has increased over the
382 past years, an effective method for monitoring infectious agents in mouse colonies kept in
383 IVC racks has not yet been established. This is because optimal sentinel monitoring must
384 conform to a wholistic approach taking into consideration technical, microbiological and
385 handling parameters (Compton *et al.* 2004a; Nicklas *et al.* 2002). In contrast to recently

386 published work with experimentally infected mice (Compton *et al.* 2004b) and probable
387 subsequent high levels of pathogen shedding, our study was aimed at evaluating a facility-
388 adapted, sentinel-based monitoring programme for naturally infected mice in IVC racks under
389 field conditions. The design of our study therefore closely resembles the situation found in a
390 majority of research rodent facilities. In our studies, sentinels exposed to soiled bedding,
391 exhaust air and a combination of both were able to detect MHV. Intestinal flagellates were not
392 detected by exhaust air only. Whereas *S obvelata* was effectively detected by a combination
393 of soiled bedding and exhaust air, MPV was not detected by the means employed in this
394 study. An important finding was the maintenance of biocontainment at the cage level in the
395 IVC racks since negative control mice were not infected throughout the experiment.
396 With respect to MHV detection by SBS, by day 28 in Experiment Q-2, only one of
397 five sentinels were positive. However, by day 42 in both Experiments Q-1 and Q-2 all eight
398 SBS were positive, indicating that MHV is effectively detected at this time point. By day 84,
399 one SBS did not seroconvert although the cage mates examined at day 42 and 84 had been
400 positive, indicating a lack of horizontal infection within the sentinel cage or absence of
401 seroconversion following infection (Casebolt *et al.* 1987). Further possible explanations
402 include virus excretion levels below the infectious dose or individual differences in
403 susceptibility to MHV infection in the outbred Swiss mice. Exhaust air monitoring by EAS
404 was as effective as soiled bedding monitoring using SBS except for the monitoring period
405 days 43 to 84 in Experiment Q-2. This may be explained by a potentially lower virus load in
406 the exhaust air as compared to the soiled bedding. MHV monitoring by CS was as effective to
407 that using SBS. From these data we recommend employment of at least two SBS for at least
408 42 days to monitor an IVC rack. Taken together, our data show that all three types of sentinels
409 were suitable for early detection of MHV. Our field study data confirm the results from
410 experimental infections (Compton *et al.* 2004b) which showed that MHV was equally well
411 transmitted by soiled bedding and by exhaust air. It is interesting to note that the use of

412 exhaust air sentinels is equally informative. In addition, it has the advantage that labour
413 intensive sampling of soiled bedding is not necessary.

414 Mouse parvovirus was not detected by the three monitoring methods although it was
415 present in index mice as shown by its transmission from IM to previously seronegative mating
416 partners as well as to offspring (Table 2). In experimentally infected mice (Compton *et al.*
417 2004b), MPV was also not detected by soiled bedding sentinels nor exhaust air sentinels
418 although it was detected by contact sentinels and on filters put into the airways of the IVC
419 rack. Also the amount of soiled bedding taken from the cages of infected mice to which
420 sentinels were exposed as well as the MPV strain used for infection were critical. Failure in
421 detecting MPV by all sentinels confirms observations from various facilities that MPV
422 detection by soiled bedding is sporadic (unpublished data). Since data from randomly
423 sampled mice is not representative of the status of an IVC rack and the use of contact
424 sentinels is not practical a suitable monitoring method for this virus still needs to be
425 established.

426 As an alternative to sentinel monitoring, the use of particle filters in the exhaust
427 airway ducts with subsequent PCR analysis provides adequate information on the MPV status
428 of mice in an IVC rack (Compton *et al.* 2004b). However, PCR tests are still costly and
429 routine particle filter analysis is not yet established.

430 From the intestinal flagellates present in index mice at high incidence in Experiment
431 Q-1 and at low incidence in Experiment Q-2, *Enteromonas* spp. were detected by both SBS
432 and CS by day 42 in Experiment Q-1 and during the monitoring period of days 1 to 84 by
433 SBS in Experiment Q-2 suggesting that *Enteromonas* spp. was easier to detect than
434 *Chilomastix* spp. In addition, since EAS did not detect intestinal flagellates we conclude that
435 the positive CS data from Experiment Q-1 are due to exposure to soiled bedding in the first
436 place. Unfortunately, no data are available on the monitoring of intestinal flagellates in IVC
437 racks. Furthermore, there appears to be no published data on the stability of protozoan cysts

438 and trophozoites in the environment or under the frequent air-change conditions present in an
439 IVC. Similarly, there is a lack of information on the modes of transmission of protozoa other
440 than horizontal infection and on their infective doses. The high air change rate in the IVC
441 would detrimentally affect the survival of flagellates and might explain the inability of EAS to
442 detect the intestinal flagellates. The observation that *Chilomastix* spp. and *Trichomonas* spp.
443 were detected less frequently than *Enteromonas* spp. may be the result of the aforementioned
444 drying effect combined with the fact that *Enteromonas* spp. was always observed in greater
445 numbers than the other two flagellates in the index mice. In addition, the other two flagellates
446 may have been excreted in numbers below the infective dose. A plausible reason for the
447 observation in Experiment 1 that Enteromonas was transmitted to sentinels by day 42, was
448 still present in the index mice at day 84 but was absent from the sentinels on day 84 could be
449 due to a low incidence of the infection in the sentinels which did not allow detection in their
450 caecal contents. Nonetheless, as shown in table 2, the data indicate that the 3-month exposure
451 period of sentinels to soiled bedding as recommended by FELASA is sufficient for detection
452 of flagellates.
453 In the GMC study, where only combined sentinels were used, *S. obvelata* was detected
454 as early as 27 days after first contact of the sentinels with soiled bedding taken from the
455 infected colony. A minimum exposure to soiled bedding for 7 days was sufficient to transmit
456 *S. obvelata*. Taking into account a life cycle of 15 days, monitoring of colonies for *S. obvelata*
457 as performed in our studies can be considered effective. After first detection of *S. obvelata*
458 infection in the GMC by necropsy during routine monitoring, additional information was
459 obtained with anal tapes. The latter method proved to be effective in both identification of
460 infected mice and subsequent non-invasive monitoring of the sentinels at one week intervals.
461 Whereas 77 % of infected mice were detected by anal tape, 91% were detected by analysis of
462 gut contents, confirming earlier observations (Flynn 1973; Huerkamp 1993). This discrepancy

463 could be due to an early stage of infection where eggs have not yet been deposited in the peri-
464 anal region or to the number of worms in the gut below the detection level.
465 We conclude that the sentinel monitoring programme implemented in the present
466 study is suitable for rapid detection of *S. obvelata* in IVC racks. If required, additional
467 analyses by anal tapes provide an additional non-invasive detailed option on a single-mouse
468 level.
469 Biocontainment is an important issue when mouse colonies with different
470 microbiological status are kept in close vicinity. Throughout the Q-study all negative control
471 mice retained their microbiological status indicating that the IVCs used in these studies
472 together with adequate husbandry procedures prevented cage-to-cage contaminations
473 throughout the experimental periods of the two Q-experiments. Moreover, in the GMC study,
474 where mice were frequently transferred from their IVCs and cage changing stations to the
475 phenotyping devices, spread of *S. obvelata* to non-infected colonies did not occur.
476 Identification of infected mice allowed eradication of *S. obvelata* from the unit without
477 therapeutic intervention by culling the infected colony, autoclaving the IVC racks, and
478 disinfecting surfaces of the floors and equipment. The absence of *S. obvelata* during the seven
479 month follow-up period showed that the eradication procedures were equally effective as that
480 observed after anthelmintic treatment (Huerkamp *et al.* 2000). However, avoiding treatment
481 of the remaining mice in the GMC was of particular importance because of potential
482 undesirable effects of anthelmintics (Blakley and Rousseaux 1991; Mohn and Philipp 1981)
483 on some of the 240 parameters being measured in the GMC.
484 Microbiological monitoring of rodent colonies is an evolving process that is
485 influenced by new developments of caging systems, husbandry refinements, new rodent
486 genotypes or newly emerging murine infectious agents. Each monitoring program should
487 therefore consider both equipment-related and infectious agent–related parameters. Taken
488 together, the use of exhaust air in addition to soiled bedding has some advantages over the use

489 of soiled bedding alone in the detection of MHV as shown in the present study and for Sendai
490 virus as reported previously (Compton *et al.* 2004b). We therefore recommend the use of
491 combined sentinels in view of increasing worldwide mouse transfers. Where infectious agents
492 are transmitted primarily by direct contact, as shown for MPV in this study, the use of
493 sentinels should be complemented by direct detection of infectious agents sampled either
494 from animals, equipment or particle filters. Exhaust air sampling on particle filters with
495 subsequent PCR analysis therefore might accentuate the full power of sentinel monitoring of
496 IVCs in the future.
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499
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501
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Table 1 Monitoring of infectious agents in IVC racks using soiled bedding and combined sentinels* in Experiment Q-1

	observation period						
	days 1-42			days 1-84			days 1-84
Infectious agent	SBS[a]	CS[b]	C[c]	SBS	CS	C	IM[d]
MHV	4/4	3/4	0/16	3/4	4/4	0/16	3/20
Enteromonas spp.	2/4	2/4	0/16	0/4	0/4	0/16	11/20
Chilomastix spp.	0/4	0/4	0/16	0/4	0/4	0/16	8/20

* combined sentinels received used bedding and exhaust air

MHV: Mouse Hepatitis Virus

a, SBS: soiled bedding sentinels

b, CS: combined sentinels (exhaust air/soiled bedding)

c, C: negative control mice used for investigation of biocontainment

d, random samples of index mice (IM)

Table 2 Monitoring of infectious agents in IVC racks using soiled bedding and exhaust air sentinels in Experiment Q-2

	monitoring period											
	days 1-28[a]			days 1-42			days 43-84		days 1-84			days 1- 84
Infectious Agent	SBS[b]	EAS[c]	C[d]	SBS	EAS	C	SBS	EAS	SBS	EAS	C	IM[e]
MHV	1/5[f]	2/5[f]	0/3	4/4	4/4	0/16	2/2	0/2	2/2	2/2	0/16	12/36[g]
MPV	0/5	0/5	0/3	0/4	0/4	0/16	0/2	0/2	0/2	0/2	0/16	3/39[g]
Enteromonas spp.	n.t.	n.t.	n.t.	0/4	0/4	0/16	0/2	0/2	1/2	0/2	0/16	4/43
Trichomonas spp.	n.t.	n.t.	n.t.	0/4	0/4	0/16	0/2	0/2	1/2	0/2	0/16	1/43
Chilomastix spp.	n.t.	n.t.	n.t.	0/4	0/4	0/16	0/2	0/2	1/2	0/2	0/16	2/43

MHV: Mouse Hepatitis Virus
MPV: Mouse Parvovirus
a, at day 28, five out of six mice were bled and tested by serology for MHV and MPV only
b, SBS: soiled bedding sentinels
c, EAS: exhaust air sentinels
d, C: negative control mice used for investigation of biocontainment
e, random samples of index mice, investigated at day 84
f, one mouse was equivocal low positive for MHV
g, seropositve mice include previously seronegative mating partners and their offspring, confirming the presence of infectious MHV and MPV in the IM
n.t., not tested

Table 3 Detection of *S. obvelata* in mice of the infected colony by anal tape and examination of gut contents in the GMC-study

Method and result of detection of *S. obvelata*		
Anal tape [a] (eggs)	Gut content [b] (worms)	No. of mice
Positive	Positive	15/60
Positive	Negative	2/60
Negative	Positive	5/60
Negative	Negative	38/60

a, two consecutive examinations at a 7 day interval

b, one examination, simultaneous with the second anal tape

15 of the 75 mice of the infected colony were not tested

Table 4 Detection of *S. obvelata* in combined sentinels* kept in IVC racks in the GMC-study

	IVC rack no				
Parameter	A2	B2	C3	D1	E1
Time of exposure of combined sentinels to soiled bedding/air of infected mice (days)	7/11	27/28	13/14	5/10	14/18
Interval from first possible infection by soiled bedding/air to positive finding (days)	38/42	36/37	27/28	27/32	14/18
Incidence (No. of infected sentinels/No. of sentinels investigated)	2/2	2/2	2/2	2/2	1/2[a]
Follow-up investigation (No. of infected sentinels/No. of sentinels investigated)[b]	0/4	0/4	0/4	0/4	0/4

* combined sentinels received used bedding and exhaust air

a, only one *S. obvelata* egg on the tape which may indicate an early infection

b, 2 subsequent investigations in 3-month intervals

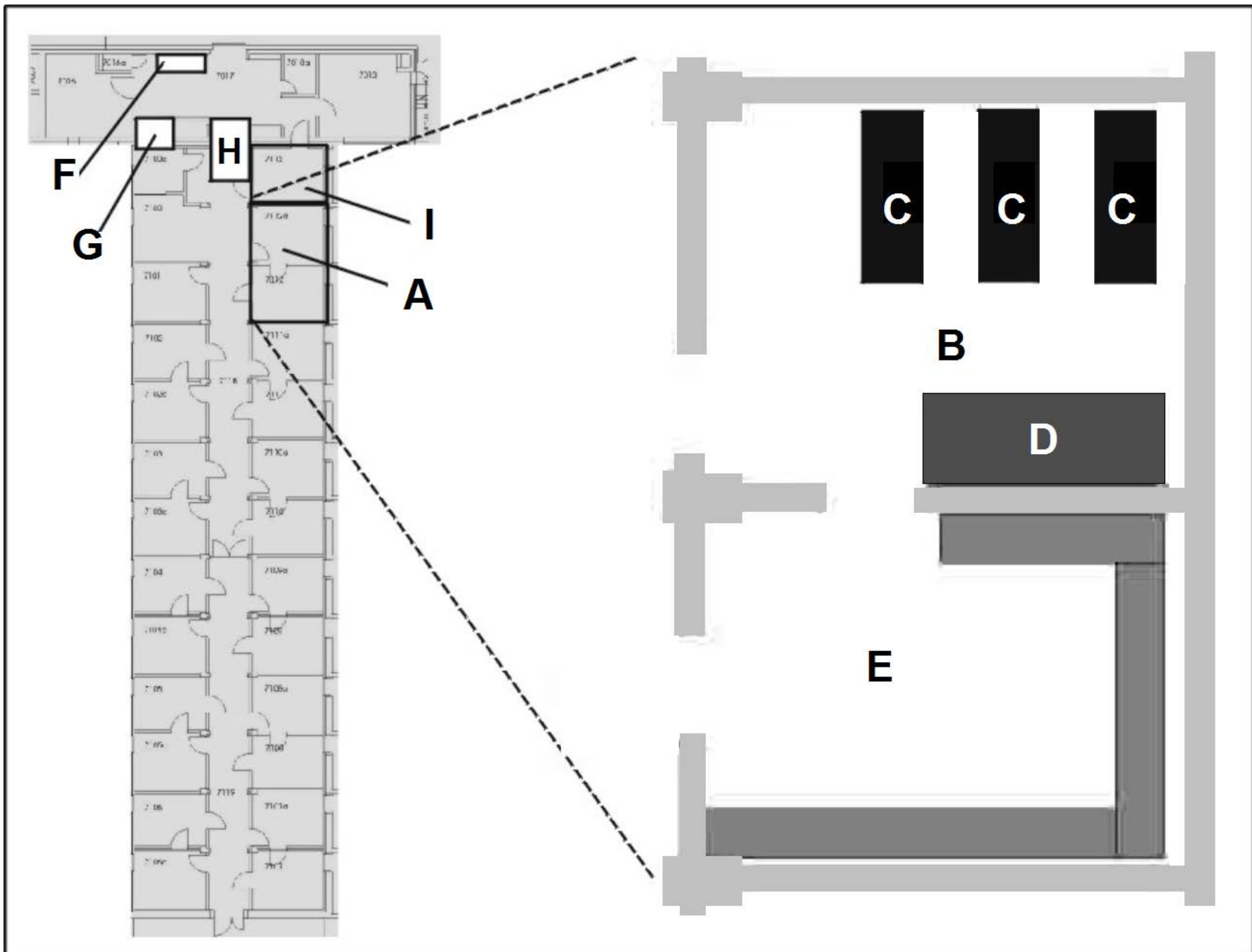

Fig. 1 Schematic view of the German Mouse Clinic (GMC)

The GMC consists of eleven modules (A), each comprising a mouse room (B), equipped with HVAC-connected IVC racks (C) and a class II changing station (D) and of an adjacent laboratory (E) equipped with devices for the phenotypical analysis of mouse mutants. Upon arrival from external facilities, mice are transferred from transport boxes into autoclaved type II cages in the class II laminar air flow import changing station (F), brought through a sluice (G) into the GMC, and placed into newly autoclaved IVC racks (C) in the respective mouse room (D) of the first module. Mice are transferred through the different modules according to the phenotyping workflow. Depending on the time required for the tests the individual colonies remain in the different modules for various periods of time.

(H) autoclave

(I) changing room

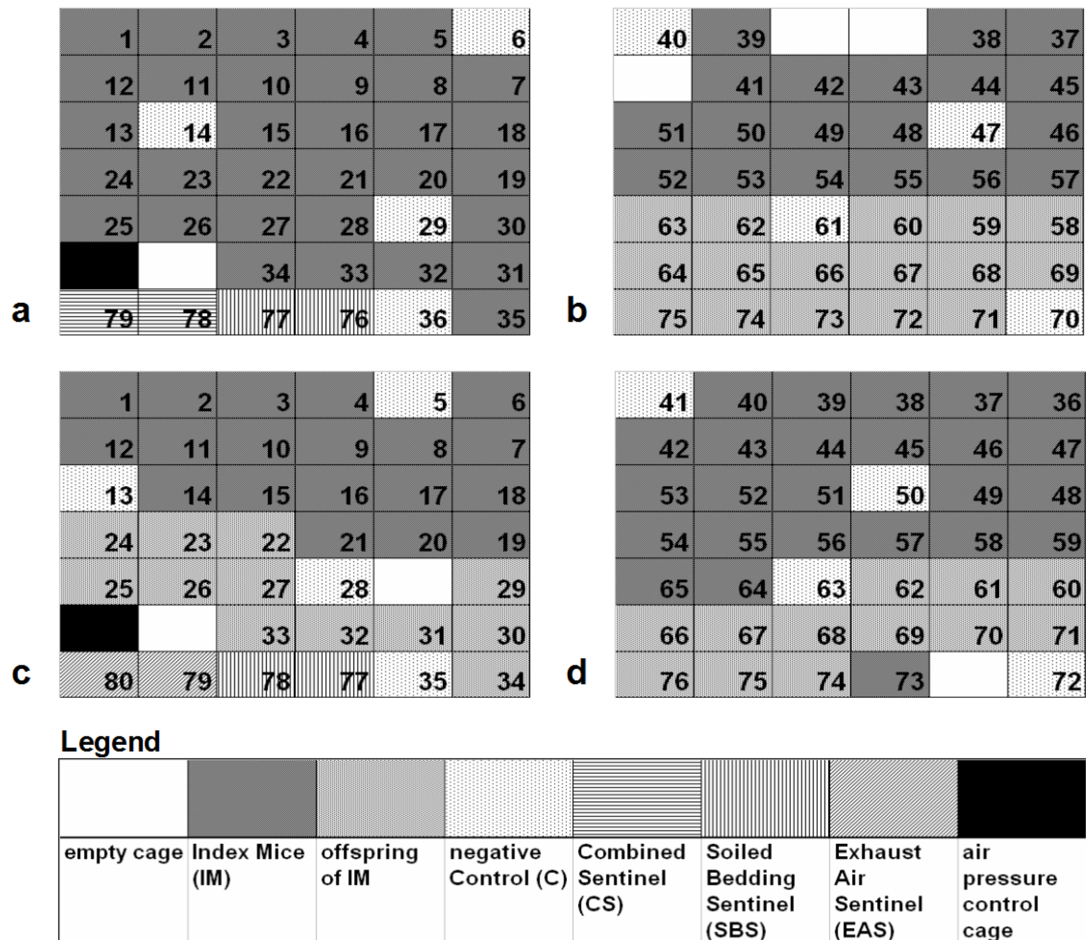

Fig. 2 Individually ventilated cage (IVC) rack conformation for Experiments Q-1 and Q-2.

a, Experiment Q-1 IVC rack front side

b, Experiment Q-1 IVC rack back side

c, Experiment Q-2 IVC rack front side

d, ExperimentQ-2 IVC rack back side

Numbers indicate the order of cage changing.

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