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Original scientific article

Effect of non-sterile bedding and feed on the spread of some infectious agents among BALB/c mice in SPF animal housing

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Summary

The aim of this study was to identify and assess the risk of spreading infectious agents in a colony of specific pathogen free (SPF) BALB/cOlaHsd mice when using non-sterile bedding and feed. The total number of microbial colonies in bedding was estimated, and their species identification was carried out using bacteriological methods. Infectious agents regulated for SPF mice were determined in the mouth and fecal swabs of mice using real time PCR. The mice in the animal facility were housed in individually ventilated cages (IVC). To assess the effect of air, one group of animals was kept in open-air cages (OAC). The study duration was 90 days. *Staphylococcus epidermidis* was identified in bedding in the groups of animals that were kept on non-sterilized bedding and in OAC. In addition, the level of bacterial contamination in the group with non-sterile bedding was significantly higher (1065 CFU/g) than the control group and higher than the other groups. These microorganisms in bedding were *Lactobacillus spp, Staphylococcus spp* and *Escherichia coli* . *Staphylococcus spp* and *E.coli* are not commensal microflora, but can be found in SPF animals. As a result of PCR testing, only *Rodentibacter pneumotropicus* was found in the mice; it was present in each group. It is assumed that the mouse colony was initially contaminated with this infectious agent. No visible pathological changes such as enlargement of the spleen, liver and lymph nodes, or intestinal distension, were detected at necropsy. The results of this study showed that personnel are possibly the main source of *S. epidermidis* because this microorganism is not found in SPF-mice. The transfer of bacterial agents occurs mainly through poor-quality bedding, as well as by the air.

Introduction

The quality of laboratory animals depends on three factors: genetic (belonging to the standard of a particular strain/stock), health status and environmental conditions. The health of laboratory animals is particularly important, and is the most difficult factor to control and manage. Some infectious agents should not be found in laboratory animals. First of all, this because these infectious agents could cause a subclinical course of the disease with serious changes in the body (immune response, tissues changes, etc.) leading to the spontaneous death of some or all the animals. This will have an impact on the quality of studies (Baker 1998; GV-SOLAS Working Group on Hygiene 1999; Marx et al. 2017). In addition, some of the infectious agents could be hazardous to humans, in particular to animal care workers. Lymphocytic choriomeningitis virus (LCMV) can be transmitted from mice to humans (Emonet et al. 2007). Monitoring the health of a colony of laboratory animals is therefore of paramount importance both for the qual-

ity of scientific studies and the health of personnel. A number of recommendations have been developed for monitoring the health of rodent and rabbit colonies in breeding and experimental units (FELASA working group 2014). The route and method of spreading infectious agents in colonies depend on numerous factors. Infection can occur from packaging boxes and non-sterile feed during transportation of animals (Lindstrom et al. 2018). The dependence of infectivity on the gender and age of the animals, and on received dose of virus, has been shown for some viral infectious agents, for example mouse minute virus (MMV) (Thomas et al. 2007). Mouse hepatic virus (MHV), mouse parvoviruses (MPV), Theiler's murine encephalomyelitis virus (TMEV), *Helicobacter* spp. and fur mites spread more efficiently through bedding than Sendai virus (de Bruin et al. 2016). However, not all infectious agents can be effectively transferred through bedding. Sudden outbreaks of parvovirus infection in colonies can be caused by non-sterile or improperly sterilized feed (Watson 2013). But, most often viral infections are transmitted through animals with unknown health status (Homberger and Thomann 1994). The variety of infectious agents and means of spread of infection in an animal colony greatly complicate the work for identifying and reducing these risks.

We believe that viruses spread most efficiently in SPF colonies mainly through animals with unknown health status, while bacterial contamination occurs through poor quality feed and bedding. The purpose of this study was to investigate the effect of non-sterile feed and bedding on the spread of some infectious agents among SPF mice.

Materials and Methods

Ethical statement

The study protocol was reviewed and approved at the IACUC meeting of the National Scientific Center of Especially Dangerous Infections (NSCEDI) No. 86/2 dated March 17, 2020.

Animal housing

The study was carried out in a separate 200-m^2 facility with 2 rooms for keeping mice and rats, as well as a storage unit for clean materials, 2 manipulation rooms and areas for washing and sterilization. The ventilation system for the rooms housing the animals was separate from that for other areas. An excess pressure of 10 Pa, a temperature of 24±2° C and 12 h light–dark cycle were constantly maintained inside the clean zone. The level of particles in the air was in accordance with ISO 14644-2 Class 7. The animal care staff routinely wore personal protective equipment: mask, gown and gloves. In barrier rooms, researchers wore a cap, mask, gloves, disposable isolation gown and shoe covers. All mice were kept in polysulfone IVC cages (396 x 215 x 172 mm, floor area: 542 cm^2) (Tecniplast, Italy). IVC cages without lids (but with a top grid) were used as open-air cages (OAC). OAC cages were placed next to IVC cages on the table. All manipulations with animals were carried out at CS5 cage change stations (Tecniplast, Italy). Cages were changed weekly. Microbiological air monitoring was regularly carried out in the animal housing rooms and manipulation rooms. The standard operating procedure provided for the sterilization of cages, feed and bedding at 121° C for 20 minutes. The welfare of laboratory animals was regularly monitored by the IACUC.

Bedding

Rehofix MK 2000 (JRS, Germany), corn granulate, was used as the bedding material. The size of the granules was 2-3 mm.

Animals

The study used 40 male and female SPF BALB/cOlaHsd mice, 5 animals per cage, weighing 21-25 g (animals, 6–12-weeks-of-age). Mice were purchased from Envigo (Netherlands). Mouse barrier rooms excluded the pathogens noted in Table 1. Prior to the study, health monitoring in the animal housing rooms was achieved through SBS serology and fecal and oral swab PCR analysis on a quarterly basis.

Feeding

V1534-300 (SSNIFF, Germany) complete autoclavable diet was used for feeding laboratory mice.

Table 1. List of pathogens excluded from the mouse barrier rooms.

Viruses	Bacteria
Mouse hepatitis virus, Mouse rotavirus (EDIM), Theiler's murine encephalomyelitis virus, Murine norovirus, Mouse parvovirus 1, Mouse parvovirus 2, Minute virus of mice	*Helicobacter spp., Clostridium piliforme, Mycoplasma pulmonis, Streptococcus pneumoniae, Streptococcus b-haemolyticus, Rodentibacter pneumotropicus*

Table 2. Experiment design.

Groups	Exposure factors	Microbiological study of bedding	No. of animals per cage/a total of animals
1. IVC, sterile bedding, feed and water	Control	Before the start, the 30th, 60th and 90th day	5/10
2. IVC, sterile feed and water	Non-sterile bedding	Before the start, the 30th, 60th and 90th day	5/10
3. IVC, sterile bedding and water	Non-sterile feed	Before the start, the 30th, 60th and 90th day	5/10
4. OAC, sterile bedding, feed and water	Open-air system	Before the start, the 30th, 60th and 90th day	5/10

IVC – individually ventilated cage; OAC – open-air cage

Study design

Experimental groups with different exposure factors and microbiological study of bedding are presented in Table 2.

The study duration was 90 days. Mouth and fecal swabs were collected from the animals for use in the PCR assay. After this, the animals were euthanized with CO_2. The animals were dissected under aseptic conditions and macroscopic examination was performed to identify visible pathologies. Samples of organs were taken and placed in a 10% solution of neutral formalin for further histological studies.

Bacteriological studies

At each sampling time point two samples of bedding from each cage were analyzed by bacteriological culture methods. For this, 10 g of each sample was homogenized with sterile water in a ratio of 1 to 10. 0.1 mL of the bedding homogenate was applied to the surface of plates with Meat Infusion agar (Himedia, India) and Sabouraud agar (Himedia, India). Baird-Parker agar (Himedia, India) and Endo agar (Himedia, India) selective media were used to identify microorganisms. Plates were incubated at 37° C for 48 h, those with Sabouraud agar at 22 °C for 5 days.

DNA extraction for PCR assay

500 µL of the Inhibitex Buffer (Qiagen, Netherlands) was added to each fecal sample and mouth swab and incubated for 15 minutes at 70 ° C (Sanyo, Japan). DNA isolation was performed according to the kit protocol (QIAamp® DNA Mini Kit, USA). The DNA concentration was determined on the Qubit 4 fluorometer (Thermo Fisher Scientific, USA) using reagents (Thermo Fisher Scientific, USA). The DNA concentration in the samples ranged from 1.17 to 33.4 ng per µL.

PCR assay

The *Helicobacter* spp, *Clostridium piliforme*, *Mycoplasma pulmonis*, *Streptococcus pneumonia*e, *Streptococcus b-haemolyticus*, *Rodentibacter pneumotropicus* (*Pasteurella pneumotropica* CCUG 12398^T), Mouse parvovirus, Mouse parvovirus 2, Minute virus of mice (BBTLAB, Russia) kits were used in the real time PCR. DNA amplification was carried out with 20 µL of the reaction mixture. The kit positive control (BBTLAB, Russia) was used. Purified water served as a negative control. Amplification program: 5 min pre-incubation at 95° C followed by 40 cycles of 15 sec at 95° C, 25 sec at 62° C, and the luminosity readout for 25 sec at 62° C. Melting curve plotting was at temperatures from 65° C to 95° C with a step of 0.5° C in 5 seconds.

Statistical analysis

Data are presented as median, minimum and maximum. CFU indices were subjected to statistical analysis using the Kruskal-Wallis test with *a posteriori* comparison of each group to the non-parametric Dunn's criterion. All calculations are carried out with the GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA). The p-value < 0.05 was considered as a significant difference.

Results

Health monitoring and studies on the effect of feed and bedding on the spread of certain infectious agents in the mouse colony were carried out in the animal facility for toxicological testing. Following the sterilization process, the microbiological purity of the feed and bedding was regularly monitored. In addition, a monthly microbiological study of bedding from each cage was carried out. Bedding samples were taken when animals were transferred from a dirty cage to a clean one. The results of counting

Table 3. Growth (median, min and max) of bacteria on Meat Infusion agar, CFU/g bedding.

Groups	Days		
	30	60	90
1 (IVC)	500 (450, 520)	267.5 (230, 310)	367.5 (280, 420)
2 (IVC)	1065 (780, 1200) *	1030 (890, 1320) *	1020 (860, 1200) *
3 (IVC)	458 (320, 530)	321.5 (230, 452)	409,5 (365, 580)
4 (OAC)	835 (650, 980)	735 (692, 1000)	650 (498, 670)
P value	0.003	0.002	0.001

Note: * p<0.05 (Kruskal-Wallis test) compared with control group 1

the total number of colonies grown on Meat Infusion agar are shown in Table 3.

Despite the fact that animals in groups 1, 3, and 4 were kept on sterile bedding, the number of microbial colonies reached 835 CFU/g bedding. It is to be expected that the number of colonies of microorganisms was always higher in the group with non-sterile bedding. This level reached 1065 log CFU/g. The current study showed that only group two with non-sterile bedding was statistically significant compared to the control group ($P < 0.05$). In addition, abundant growth of *Lactobacillus* species was observed in all groups.

Selective media were used to identify gram-positive *Staphylococcus* species and enterobacteria. The identification results are presented in Table 4.

Most of the microorganisms found in bedding were identified as *Staphylococcus epidermidis*; *S. aureus was not detected*. Moreover, *S. epidermidis* was detected only when using non-sterile bedding or in OAC. *Escherichia coli* were also identified using Endo agar.

At the end of the experiment, the mouth and fecal swabs were collected from all animals for PCR testing. The results are shown in Table 5.

As a result of PCR testing, only *Rodentibacter pneumotropicus* was found in mice and was present in each group.

No visible pathological changes such as enlargement of the spleen, liver and lymph nodes, or intestine distension, were detected at necropsy. Histological analysis showed that the morphology of these organs was not different between the groups of mice.

Table 4. Species composition of bacteria grown from bedding on selective media (CFU/g).

Groups	Selective medium	
	Baird-Parker agar (*Staphylococcus spp.*)	Endo agar (*Escherichia coli*)
30 days		
1 (IVC)	-	33
2 (IVC)	323	158
3 (IVC)	-	127
4 (OAC)	230	160
60 days		
1 (IVC)	-	80
2 (IVC)	320	30
3 (IVC)	-	60
4 (OAC)	90	100
90 days		
1 (IVC)	-	190
2 (IVC)	140	240
3 (IVC)	-	180
4 (OAC)	180	260

Table 5. Results of PCR testing of samples for infectious agents

Infectious agents	Groups/No. mice with positive PCR results*			
	1	2	3	4
Helicobacter spp.	0	0	0	0
Clostridium piliforme	0	0	0	0
Mycoplasma pulmonis	0	0	0	0
Streptococcus pneumoniae	0	0	0	0
Streptococcus b-haemolyticus	0	0	0	0
Rodentibacter pneumotropicus	2 of 10	4 of 10	6 of 10	4 of 10
Mouse parvovirus 1 (MPV-1)	0	0	0	0
Mouse parvovirus 2 (MPV-2)	0	0	0	0
Minute virus of mice (MVM)	0	0	0	0

Note: the total number of mice tested is 40

Discussion

The results obtained are of particular interest for organizing health monitoring (HM) in SPF animal housing. Unsterilized bedding was one of the major sources of contamination with common microflora, since the total number of microorganisms CFU/g was higher in group 2 than after keeping animals on sterile bedding in other groups. In this study, *Staphylococcus epidermidis* was identified in non-sterile bedding. Perhaps this indicates the personnel as the source of bacteria, because this species is not typical for mice and is found only on human skin (Nagase et al. 2002; Tavakkol et al. 2010). Although, other researchers have shown that *S. epidermidis* exist in the female reproductive tract of mice (Ono et al. 2015). Anyway, *S. epidermidis* in SPF-animals can affect the production of cytokines by skin cells (Kosiewicz et al. 2013). The discovered bacteria of the genus *Lactobacillus* belong to the commensal microflora of mice. Animals with SPF status in the first group had significantly fewer *E. coli* of the total bacterial population only on day 30. *E. coli*, although not related to the commensal microflora and can sometimes cause pathology (Treuting et al. 2012), is often isolated when monitoring the health of animal colonies (Benga et al. 2014). In general, the bacterial abundance in SPF animals is much lower than in conventional animals (Wu et al. 2018). The mouse microflora is characterized by the predominance of the types (phyla) *Firmicutes* and *Bacteroidetes*, which include *Lactobacillus* (Ley et al. 2005). However, the detection of microorganisms and counting of their number in bedding may only indicate the general level of compliance with aseptic and hygiene rules when keeping laboratory animals. In HM, the PCR assay plays a dominant role, as it allows detecting the genetic material of infectious agents, some of which are poorly , or not at all, isolated by culture methods. In addition, conventional bacteriological methods are costly and time-consuming, especially for routine use (Fahey and Olekszak 2015). These microorganisms include *Rodentibacter pneumotropicus*, the number of which, when isolated from an animal's body, may be insufficient to obtain colonies on media (Ouellet et al. 2011). *R. pneumotropicus* was detected by PCR in mice of all groups. Other bacteria, namely *Helicobacter* spp., *C. piliforme*, *M. pulmonis*, *S. pneumonia* and *S. b-haemolyticus* were not detected This allows us to make the assumption that there was no source of contamination inside the SPF colony, or its effect was not enough to spread these microorganisms in the colony (Bohr 2006; Liang et al. 2009). This is especially true for OAC group 4. The situation was also similar with the MPV and MVM viruses. These viruses have a high level of spread in the colonies of laboratory animals and are capable of causing subclinical diseases (Carty 2008; Filipovska-Naumovska et al. 2010). The spread of certain pathogenic bacteria and viruses in animal colonies depends on the quality and efficiency of cage washing (Compton and Macy 2015). The spread of viral infections to a lesser extent depends on the use of personal protective equipment (PPE), although the complete absence of PPE is not acceptable (Baker et al. 2014). In addition, efficient operation of heating, ventilation and air-conditioning (HVAC) systems significantly reduces the risk of spreading infectious agents in an animal house (Goyal et al. 2011). Based on the results obtained, several conclusions can be drawn. One of the routes of transmission of bacterial pathogens is non-sterile or poorly sterilized bedding, as well as airborne transmission, as has been shown in this study. The source of *S. epidermidis* could be staff caring for the animals because this microorganism is not typical for mice and frequently colonizes human skin (Otto 2009).

The combination of an IVC system for keeping animals and maintaining indoor air according to ISO-14644 class 7 more effectively protects against bacterial contamination than the use of OAC with the same indoor air quality.

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Conflict of interest

The authors declared no potential conflict of interest.

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