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Critical remarks on the use of environmental samples for the determination of the infectious status in laboratory mouse animal facilities

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Introduction

To ensure reproducibility of animal experiments standardized conditions in animal facilities are required. In particular, the microbiological quality of animals can critically influence animal welfare, the validity and reproducibility of research data, and ensures protection against zoonosis. Therefore, health surveillance of animals in breeding and experimental mouse facilities is crucial. FELASA published recommendations concerning the use of animals for testing, samples to test, frequency of sampling, commonly used test methods, and interpretation of results (Mähler et al., 2014).

To date, health monitoring of mouse colonies is performed primarily by testing of animals. For this purpose, retired breeders, colony animals as well as sick and dead colony animals or sentinels are used. In colonies housed in individually ventilated cages (IVC), sentinel animals are preferably used to detect infectious agents due to the system's intrinsic characteristics of biocontainment and bioexclusion[1]. Therefore, either soiled bedding is transferred to sentinel animals during routine cage changing (soiled-bedding sentinels) or sentinel animals are kept in direct contact with colony animals (contact sentinels) in many different variations and combinations. Animals are tested after an exposition period of usually 10 to 12 weeks and samples are taken for microbiological and parasitological examination (necropsy, microbial culture, microscopy, serology, and PCR). Also, samples taken directly from sentinel or colony animals e.g., oral swabs, genital swabs, fur samples, fecal pellets, and blood samples can be used for diagnosis (Henderson et al., 2013). Furthermore, environmental samples e.g., dust swabs from cages or the exhaust air ventilation tubing or plenum, exhaust air filters or dedicated capture media from rack air ventilation systems, commonly referred to as exhaust air dust (EAD) can be used for health monitoring (Compton et al., 2004a, Jensen et al., 2013, Miller and Brielmeier, 2017, Mahabir et al., 2019).

The advent of the use of IVC systems, which provide biocontainment at the cage level (Compton et al., 2004a, Compton et al., 2004b, Brielmeier et al., 2006), was paired with an increased challenge of reliable health monitoring. Each cage must be considered an independent microbiological unit since IVC housing reduces the spread of infectious agents among cages.

A vast range of possibilities on how to conduct health monitoring in mouse facilities is available today. Therefore, decision-making as to which approach to take, might not be easy. Many approaches claim to be outstanding regarding sensitivity, costs, 3R, and workload. To support laboratory animal veterinarians and animal facility managers in designing effective health monitoring programmes, current data are compiled in this overview including some critical remarks concerning interpretation of the results

Sentinel testing

It is well known that the detection of infectious agents in mouse facilities via soiled bedding sentinels is dependent on a variety of factors such as prevalence of infectious agents, mode of transmission, infectious dose, duration and intensity of shedding, resistance of pathogens to environmental conditions, sentinel animal (strain, age, soiled bedding or contact sentinels) as well as the frequency and amount of soiled bedding transferred. For example, there is a risk

[1] http://www.gv-solas.de/fileadmin/user_upload/pdf_publikation/Hygiene/20151023hyg_ivc.pdf

of diluting the infectious dose when pooling bedding from many cages and thus the number of agents transmitted may be too low to achieve infection and seroconversion. Infectious agents known to be transmitted via dirty bedding are e.g., mouse hepatitis virus (MHV), mouse parvovirus (MPV), murine norovirus (MNV), *Helicobacter* spp., *Clostridium piliforme*, and pinworms (Waggie et al. 1984, Gibson et al. 1987, Thigpen et al. 1989, Dillehay et al. 1990, Motzel and Riley 1992, Smith et al. 1993, Whary et al. 2000, Brielmeier et al. 2006, Compton et al. 2004b, Smith et al. 2007, Manuel et al. 2008). However, agents such as Sendai virus, lymphocytic choriomeningitis virus (LCMV), *Pasteurellaceae*, *Streptobacillus moniliformis*, *Mycoplasma* spp., *Spironucleus muris*, and mites are not reliably detected in sentinel animals (Thigpen et al., 1989, Artwohl et al., 1994, Dillehay et al., 1990, Scharmann and Heller, 2001, Ike et al., 2007, Perdue et al., 2008, Lindstrom et al., 2011, Miller et al. 2016, Körner et al. 2019, Buchheister et al. 2020). Hence, it is recommended to complement routine health monitoring conducted with sentinels by other methods[2].

Environmental sampling

Currently, in animal facilities there is an increased interest in the use of polymerase chain reaction (PCR) testing of environmental samples for routine health monitoring. These include environmental swabs from cages or exhaust air ventilation tubing or plenum, exhaust air filters or capture media from rack air ventilation systems. Some vendors even developed special capture media to meet the user's need for easy handling.

Henderson et al. (2013) demonstrated the efficacy of in-line particle capture in IVCs by using PCR. In recent years, an increased number of reports demonstrate the use of real-time PCR to detect murine infectious agents in exhaust air particles from different IVC systems (Jensen et al., 2013, Bauer et al., 2016, Manuel et al., 2016, Miller et al., 2016a, Miller et al., 2016b, Miller and Brielmeier, 2018, Kapoor et al., 2017, Zorn et al., 2017, Körner et al., 2019, Mahabir et al., 2019, Mailhiot et al., 2020). Many of these reports show that the speed of detection is higher with the use of testing environmental samples (exposure time 1 until 4 weeks) compared to soiled bedding sentinels which usually need a longer exposure time (Jensen et al., 2013, Bauer et al., 2016, Miller et al., 2016a and 2016b, Kapoor et al., 2017, Miller and Brielmeier, 2018, Zorn et al., 2017). In two comparative studies, the rate of detection e.g., of *Helicobacter typhlonius*, *Klebsiella oxytoca*, *Rodentibacter pneumotropicus*, other *Pasteurellaceae*, MNV, murine astrovirus (MuAstV), *Tritrichomonas*, *Entamoeba*, pinworms, and fur mites was higher compared to testing animals of the respective racks (Miller and Brielmeier, 2018, Schmelting et al., 2019). However, it is also reported that some agents could not be detected in all cases via environmental sampling, e.g., MNV, mouse rotavirus, MuAstV, *Helicobacter hepaticus*, *Klebsiella oxytoca*, *Rodentibacter pneumotropicus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, ß-hemolytic streptococci (group B), and *Aspiculuris tetraptera* (Compton et al., 2004b, Ouellet et al., 2011, Leblanc et al., 2014, Bauer et al., 2016, Kapoor et al., 2017, Miller and Brielmeier, 2018, Mahabir et al., 2019). For further reading, a current comprehensive overview of PCR testing of environmental samples for the determination of the infectious status in laboratory mice is shown in Table 1. It should be noted that the results in the table are in part differing most likely due to the experimental study design.

[2] For more information see:
http://www.gv-solas.de/fileadmin/user_upload/pdf_publikation/Hygiene/2020.01Validity_health_reports.pdf
Hygienic monitoring of mice and rats in various housing systems

Advantages and disadvantages of testing environmental samples

High sensitivity and detection speed are the foremost advantages of environmental testing via PCR (Miller and Brielmeier, 2018, Durand et al., 2019, Schmelting et al., 2019, Wiese et al., 2019). Exhaust air particle-PCR reliably detected *Helicobacter hepaticus*, *Rodentibacter pneumotropicus*, and *Myocoptes musculinus* within one week at a minimal prevalence of 1/63 cages and MuAstV at a prevalence of 3/63 cages housing infected mice (Miller et al. 2016a, Miller et al., 2016b, Körner et al., 2019). Furthermore, infectious agents that are difficult to detect with traditional culture methods can be easily identified by PCR.

The number of infectious agents necessary to cause infection in animals is considered to be less relevant in environmental PCR testing as lower amounts of agents and even non-infectious organisms are detectable. Thus, agents causing only transient infections can be found in environmental samples.

When using environmental testing the mode of transmission of agents is not relevant for obtaining results (Thigpen et al., 1989, Lindstrom et al., 2011).

As a contribution to 3R, animals are not needed for detection of agents, hence maintenance and shipment of animals for diagnostic purposes is obsolete (Miller et al., 2018, Wiese et al., 2019). Especially the use of immundeficient sentinels does not make sense anymore as these animals may suffer from disease or even die. Furthermore, there is no risk of introducing infectious or opportunistic agents into colonies by sentinel animals (Dafni et al., 2019). In addition, commercially available customized capture media guarantee easy handling with minor needs for training of the respective staff and lower risk of contamination. As a side effect, costs related to purchase or production and maintenance of sentinels are reduced (Wiese et al., 2019).

On the other hand, health surveillance via environmental sampling can also have some disadvantages. When using exclusively molecular biological methods it is only possible to detect agents, which are tested for with the specific primers used. New or mutated organisms might not be detected and hence lead to false-negative results. In this case, animals are irreplaceable as they give additional information concerning clinical signs and pathology (Humbert et al., 2019). In the case of positive results, it is unknown whether nucleic acid detected in environmental samples relates to infectious organisms. Confirmatory tests using mice (e.g., pathology, bacteriology, or parasitology) might be necessary to determine, whether there is an infection ongoing in the hygienic entity and to identify cages housing the infected mice (for elimination or treatment) as it was demonstrated for fur mites and pinworms (Miller and Brielmeier, 2018). Furthermore, before starting to test by environmental sampling the absence of residual DNA needs to be confirmed. Hence, cleaning, autoclaving, and decontamination of racks, air handling units, and equipment is mandatory (Bauer et al., 2016, Manuel et al., 2016, Miller and Brielmeier, 2018, Mahabir et al., 2019). For this, redundant equipment is necessary, which, as well as baseline testing of washed and decontaminated rack systems, contributes considerably to workload and costs. Since this is difficult to achieve for subsequent health monitoring periods, in some animal facilities the equipment is only decontaminated to confirm the absence of infectious agents if infected mice have been treated or eliminated (Miller and Brielmeier, 2018).

The method which is suitable for removal of residual nucleic acids needs to be validated beforehand. When collecting environmental samples in the animal facility, there is a risk of sample contamination since methods to detect nucleic acids are highly sensitive. If dirty filters are cleaned regularly, care has to be taken to avoid cross contamination through vacuum cleaning. Hence, this can contribute to false-positive results.

Animal and cage numbers as well as rack model and bedding type influence the amount of dust generated. To date, there are no data available on the quantity ratio of infectious agents to dust, the amount of dust reaching the filters/capture media, saturation effect on filters/capture media or influence of filter type. For sampling, the type of IVC systems must also be taken into consideration. When using IVC systems with filtration at the cage level, samples must be taken from individual cages or cage filters. Furthermore, detection of agents via environmental sampling seems to be strongly dependent on the quality of the diagnostic laboratory (Wiese et al., 2019) and on the procedure of sampling. Methods to extract nucleic acids from environmental samples are crucial, as inhibitors of the polymerase enzyme are usually present in environmental samples, which may have a severe impact on the sensitivity of testing. It is therefore important that nucleic acid extraction and PCR tests are carefully validated. It is recommended to store backup samples for confirmatory tests (Miller and Brielmeier, 2018).

Conclusion

In summary, interpretation of health monitoring results becomes more complex as a variety of different methods is used by animal facilities. First, for proper interpretation of health monitoring results a detailed description of the health monitoring programme including the sampling methods used in an animal facility is crucial.

Diagnostic methods such as bacteriology, serology, parasitology, and pathology in combination with molecular biological methods are still state-of-the-art. Especially traditional methods are crucial to enable detection of new agents. Results obtained by environmental sampling should be carefully interpreted to identify potential sources of infections. In addition, confirmatory tests and sampling plans should be in place. To date, proper recommendations on frequency of environmental sampling cannot be given as available data differ to a great extent.

For environmental monitoring, the amount of data available is steadily increasing. However, it has to be taken into consideration that data for health monitoring via environmental sampling as well as sentinel animals are not available yet for all relevant agents. Hence, health monitoring solely based on sentinel testing or environmental testing is currently not advisable. Further studies still need to demonstrate the superiority of environmental samples or testing of animals for the detection of all relevant agents. This is challenging, since the prevalence of some agents in modern laboratory mouse facilities is very low.

As the traditional approach, based on the examination of sentinel and colony animals, as well as environmental microbiological monitoring alone can result in false-negative or false-positive results, a combination of various available methods (sentinel, surplus colony and sick animals, environmental samples) is recommended to increase the detection rate of unwanted microorganisms.

With the help of experts that are responsible for health monitoring, each facility should identify the most suitable method for obtaining reliable health monitoring results to protect the animal facility from infectious outbreaks and hence obtain robust research data. However, even when using a combination of methods there is still a risk of not detecting infectious agents present in the animal colony.

Table 1. Overview of PCR testing of different environmental samples for the determination of infectious agents in laboratory mice. It should be noted that the results are in part differing most likely due to the experimental study design

Infectious agent	**Swab**						**IVC rack**					
	Bedding disposal cabinet / laminar flow hood		Cages		Rack		Gauze filter in front of pre-filter		Filtrete 1500 HVAC filter in front of pre-filter		Commercially available filter	
Viruses												
Mouse hepatitis virus	+	Compton and Macy, 2015	+	Compton and Macy, 2015			+	Compton et al., 2004b	+	Bauer et al., 2016		
Mouse rotavirus							-	Compton et al., 2004b				
Murine norovirus			+	Dubelko et al., 2018*	+	Pettan-Brewer et al. 2020	+ +	Zorn et al., 2017 Miller and Brielmeier, 2018	-	Bauer et al., 2016	+ +	Mailhiot et al., 2020 Pettan-Brewer et al. 2020
Mouse parvovirus	+	Compton and Macy, 2015	+ +	Compton and Macy, 2015 Macy et al., 2009			+	Compton et al., 2004b	+	Bauer et al., 2016	+	Schäfer et al., 2019
Lymphocytic choriomeningitis virus											+	Schäfer et al., 2019
Mouse adenovirus type 2 (K87)			-	Compton and Macy, 2015								
Pneumonia virus of mice			+	Wagner et al., 2003								
Sendai virus			+ +	Wagner et al., 2003 Compton et al., 2004b			+	Compton et al., 2004b				
Murine astrovirus			+	Compton et al., 2017	+	Compton et al., 2017	+	Korner et al., 2019				
Bacteria												
Helicobacter spp.			+ +	Compton and Macy, 2015 Dubelko et al., 2018*	+	Pettan-Brewer et al. 2020	+	Miller and Brielmeier, 2018	+	Bauer et al., 2016	+ + +	Mahabir et al., 2018 Mailhiot et al., 2020 Pettan-Brewer et al., 2020
Helicobacter bilis							+	Miller and Brielmeier, 2018				
Helicobacter ganmani											+	Mailhiot et al., 2020
Helicobacter hepaticus							+ + -	Miller et al., 2016a Miller and Brielmeier, 2018 Compton et al., 2004b			+	Mahabir et al., 2018 Mailhiot et al., 2020
Helicobacter mastomyrinus												Mailhiot et al., 2020
Helicobacter muridarum							+	Compton et al., 2004b			+	Mahabir et al., 2018
Helicobacter typhlonius							+	Miller and Brielmeier, 2018			+	Mahabir et al., 2018 Mailhiot et al., 2020
Rodentibacter pneumotropicus			+	Dubelko et al., 2018*			+ +	Miller et al., 2016b Miller and Brielmeier, 2018	+	Bauer et al., 2016	+	Mahabir et al., 2018 Mailhiot et al., 2020

Other Pasteurellaceae							+	Miller and Brielmeier, 2018				
Corynebacterium bovis			+	Manuel et al., 2017	+	Manuel et al., 2016						
					+	Manuel et al., 2017						
Klebsiella oxytoca							+	Miller and Brielmeier, 2018				
Mycoplasma pulmonis	+	Compton et al., 2015										
	+	Compton and Macy, 2015										
Proteus mirabilis							+	Miller and Brielmeier, 2018				
Pseudomonas aeruginosa							-	Miller and Brielmeier, 2018				
Staphylococcus aureus							+	Miller and Brielmeier, 2018				
Fungi												
Pneumocystis murina							+	Miller and Brielmeier, 2018				
Endoparasites												
Pinworms			+	Compton and Macy, 2015					+	Bauer et al., 2016		
Aspiculuris tetraptera			+	Gerwin et al., 2017	+	Kapoor et al., 2017	+	Miller and Brielmeier, 2018				
			-	Leblanc et al., 2014	+	Leblanc et al., 2014						
Syphacia obvelata	+	Compton and Macy, 2015	-	Compton and Macy, 2015								
			+	Gerwin et al., 2017								
Entamoeba muris			+	Dubelko et al., 2018*					+	Bauer et al., 2016		
Entamoeba spp.							+	Miller and Brielmeier, 2018			+	Mahabir et al., 2018
Spironucleus muris			+	Dubelko et al., 2018*								
Trichuris muris									+	Bauer et al., 2016		
Tritrichomonas spp.							+	Miller and Brielmeier, 2018				
Ectoparasites												
Fur mites									+	Bauer et al., 2016		
Myocoptes musculinus	+	Compton and Macy, 2015	+	Gerwin et al., 2017			+	Miller and Brielmeier, 2018				
							+	Korner et al., 2019				
Myobia musculi			+	Jensen et al., 2013	+	Jensen et al., 2013						
			+	Gerwin et al., 2017								
Radfordia affinis			+	Jensen et al., 2013	+	Jensen et al., 2013					+	Pettan-Brewer et al. 2020
			+	Gerwin et al., 2017								
			+	Pettan-Brewer et al. 2020								

+: infectious agent detected, -: infectious agent not detected, HVAC: heating, ventilating, and air-conditioning system, *: filter within lid of cage

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