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Specific pathogen-free (SPF) animal status as a variable in biomedical research: Have we come full circle?

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> The germfree animal is free of all microorganisms, whereas the disease-free animal may be described as one free of pathogens and the clinical signs that they produce. … Suggested definitions are: "animals free of commonly occurring pathogens and parasites," "specific pathogen-free," "pathogen-free," "Caesarean-derived," and "disease-free" animals.
>
> [Henry L Foster (1959)]

In this commentary, we discuss the pros and cons of using specific pathogen-free (SPF) animals in biomedical research, and present individual cases where altering the gut microbiome has dramatically changed the animal's basic physiology, immune/inflammatory functions and susceptibility to infection and disease. We argue that SPF manipulation of the microbiome-host relationship has itself become a confounding variable in biomedical research, which could have major implications to human translation.

1. Brief history

In the early 1960s, researchers were increasingly frustrated with the presence of disease or infection as an unwanted variable in their experiments [1]. A mandate led to new practices and guidelines for animal housing and husbandry, including those from the National Institutes of Health (NIH) in 1963. One breeding strategy was a 'germ-free' colony that was free of ALL measurable microorganisms, including those typically found in the gut [1]. These animals were useful only if they remained in their sterile 'bubble' because on transfer to a normal environment they became ill or died. The next strategy was to breed healthy animals free of selected pathogens, from germ-free stock or Caesarean aseptic techniques, then expose the colony to an environment free of *infectious* organisms (not all) that may otherwise interfere with research goals and objectives [1].

Today, this latter SPF method of breeding is adopted by most commercial and institutional animal husbandry facilities, with certification that the colony is free from a selection of common pathogens the species is *exposed to in the wild* [1,2]. Commonly excluded enteric and respiratory bacterial pathogens include *Clostridium piliforme*, *Salmonella spp*, cilia-associated respiratory (CAR) *Bacillus*, *Mycoplasma pulomonis*, and *Citrobacter rodentium* [3]. However, differences exist among breeding facilities. For example, Charles River and Taconic Biosciences do not exclude opportunistic organisms beta-hemolytic *Streptococcus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella* spp. and *Helicobacter* spp. [4], whereas they are excluded at Harvard Medical School [5], Cornell University, and our own Australian Institute of Tropical Health and Medicine. These SPF methods are in direct contrast to breeding conventional healthy animals (sometimes called 'dirty' animals) in open cages in a controlled, health-monitored and more natural environment of antigenic exposures and indigenous gut flora.

2. Growing concerns

Concerns with SPF versus wild animals has a long history. In the early-to-mid 1960s, microbiologist Rene Dubos and colleagues studied germ-free, SPF and 'normal' wild adult mice from the same genetic origin, and were among the first to show that changes in microbiota were associated with differences in growth rate, efficiency in utilizing food, social interactions, maternal care, resistance to infection and toxins, immune function and stress [2]. Moreover, when germ-free or SPF mice were housed with normal mice these changes reverted back to their 'normal' states. Dubos further reported that unlike 'normal' wild mice, their SPF animals lacked facultatively anaerobic gram-negative bacteria, such as *Escherichia coli*, in their gut microbiota [2].

In 1965, Dubos's colleague Russell Schaedler attempted to standardize the practice by breeding mice with more conventional microbiota to represent a natural state [6]. Schaedler colonized animals with two *Lactobacillus* spp., an anaerobic *Streptococcus* spp. (group N), a strain of *Bacteroides*, an *Enterococcus* spp., and a coliform strain, all of which were isolated from Nelson Collins Swiss mice [6]. This microbial community became known as "Schaedler flora", and was supplied to animal vendors for use as a base microbiota for newly derived germ-free rodents. This pioneering work is rarely cited in the biomedical literature. In 1978, Schaedler's PhD student, Roger Orcutt was asked by the National Cancer Institute at NIH to develop a "refined microbiota" for their contract suppliers (e.g., Charles River Laboratories, Taconic Biosciences, Harlan Laboratories and Simonsen Laboratories) [7]. Today, Orcutt's approach continues to be used in many SPF facilities around

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the world to establish a "defined microbiota" prior to introduction into barrier production [7].

Nearly 40 years later, a landmark study of Beura and colleagues reported that 'standard' SPF adult mice have "immature" immune systems, and were more prone to infection than wild mice [8]. They further showed that co-housing SPF animals with pet store mice reversed the problem, and produced mice with immune systems closer to adult humans [8]. Similarly, Rosshart and colleagues showed SPF-type mice reconstituted with natural microbiota exhibited reduced inflammation and increased survival following influenza virus infection, and displayed improved resistance against colorectal tumorigenesis [9].

3. Translational gap

SPF gut microbiome heterogeneity may also contribute to the disconnect between animal studies showing promising drug development and failure to translate to humans [10]. Problems with pre-clinical modelling of human diseases was anticipated over 15 years ago by the USA Food and Drug Administration (FDA) who recommended that: "strengthening and rebuilding the disciplines of physiology, pharmacology and clinical pharmacology, will be necessary to provide the capacity to develop and evaluate new biomarkers and bridge across animal and human studies" [11]. This FDA capacity-building, Critical Path Initiative remains an ongoing challenge, and highlights another issue regarding the use of SPF animals and the concept of reductionism in basic science and its relevance to humans. *The price we pay for using "Omics" technologies to drill deeper and deeper into life's hidden secrets is often at the expense of whole body systems analysis*. Students of biomedicine need to appreciate that probing the underlying mechanisms of how drugs affect cells or tissue culture is only one tiny step toward understanding how they will behave inside a living organism. Differences in SPF colony management and what pathogens are excluded (and included) are rarely mentioned in discussions on why promising experiments in small animal models often do not translate in human trials [12,13].

4. Recommendations

We are not recommending introducing wild or pet store animal into a standard breeding colony at the risk of transmitting infectious microorganisms to resident animals or exposure of personnel to zoonotic agents. We are suggesting, that *if translation to humans is the endgame, the best chance will be from a breeding colony that mimics a more natural state in a controlled animal facility with routine microbiome profiling, standard health screening and codified ethical practices*. Different SPF, germ-free and transgenic strains may be ideally suited for mechanistic studies, with changes in the gut microbiome being used as a tool to investigate the pathogenesis of specific diseases. However, these animal models may lack translation potential if the microbiome is far removed from a natural state, and a conventionally-bred species may be required. To be fair, one could also argue that using a naturally bred colony of animals could introduce wide variability in gut microbiota and confound the research results. Finding the right balance is the key to translation.

In order to address the challenging questions of SPF definition and heterogeneity for a particular animal species or research objective, we propose the following recommendation which could accompany a *Data Availability Statement* at the end of a scientific publication.

Animal microbial/pathogen exclusion status: A list of pathogens excluded in animals supporting the conclusions of this study are available by contacting the author(s) and/or institutional data hub (with an appropriate URL).

This proposal could be mandated by journal editors, and may represent an important first step toward improving reproducibility and replicability in animal models for translational biomedical research.

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Conflict of interest statement

There are no conflicts of interest to declare.

Authorship

GPD, HLL, JLM and EB contributed equally to literature search, study design, data interpretation and writing of the manuscript.

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