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Establishment of Diagnostic Laboratory and Screening Strategy for Rodent Pathogens

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Abstract

Health monitoring surveillance is an essential requirement of barrier facility as animal research is largely depends on health status of laboratory animals being used. Early identification of pathogens by using ideal methods can prevent infections; otherwise confounding variables can occur in the experimental results of biomedical research. Syngene has been importing animals from at least 12 different barriers facilities from USA/Europe/India to conduct planned experiments at appropriate ages. However, the limiting factor was stringent quarantine period (upto 21 days), which further increases age of the animals. In order to maintain the healthy animals, comprehensive testing strategies have been implemented in a programmed manner to identify infectious agents during early stages, thereby preventing the spread of contamination to other areas within the vivarium. The primary aim was to screen representative samples of each batch as per the schedule apart from established sentinel program which were further analyzed at 6, 12 and 18 weeks interval including comprehensive annual testing. Based on the inherent challenges, a diagnostic laboratory was established at site to screen pathogens using validated methods primarily by ELISA, IFA to detect antibodies and PCR for nucleic acid detection as well as rapid screening which has been a prerequisite for early quarantine release of animals for experiments. Alternatively, MFIA has been used as high throughput assay for rodent pathogens at periodical intervals along with other methods. In addition, bacterial pathogens if any can be identified by using VITEK apart from the routine contact plating of swab samples taken from animals. The in-house diagnostic laboratory including at least 4 outside laboratories have been employed for weekly and/or sentinel samples screening by serological assays such as ELISA, MFIA, IFA and molecular technique of PCR for rodent pathogens. Collectively, the robust screening systems at multi-site laboratories which eventually reduced turnaround time by adopting high throughput assay including confirmatory methods to obtain consistent and reliable results of rodent pathogens.

Key words: Diagnostic laboratory, Screening, laboratory pathogens**,** ELISA, PCR, MFIA, Contact plating.

Introduction

Maintenance of laboratory animals with clean health status is an important aspect of barrier facilities as outcomes of experiments are mainly depends on the quality of animals used in the programs. In general, vivarium facilities ensure the animal quality by several diagnostic methods commonly used to screen viruses, mycoplasma and other organisms. Serology is considered as primary screening and indirect enzyme-linked immunosorbent assay (ELISA) has been the main diagnostic method for laboratory animals (Wunderlich *et al* 2011) which includes viruses, *Mycoplasma* spp and *Encephalitozooan cuniculi.* Periodic screening of rodents used in research is necessary to understand its health conditions to avoid unwanted infections and consequences if any. Therefore, health monitoring surveillance is indispensable and required

to focus on sample size as well as testing frequency (Clifford 2001, Pritchett-Corning 2009). The viral, bacterial infections as well as parasitological infestations of rodents used in biomedical research continue to manifest despite of improved sanitization and housing conditions (McInnes *et al* 2011). Moreover, institutions can devise a plan for testing regimen based on the risks involved either to monitor highly infectious agents often or less frequently for rest of the lower infectious agents (Pritchett-Corning 2009). The presence of pathogens in laboratory animals can lead to confounding results (Collins 1972, Nicklas 1993, Baker 1998, Livingston 2003, Blank, 2004). The misleading results may be because of either contamination might have occurred within the facility or from outside source while in transport that potentially impacts overall health conditions of animals. This may even compromises expensive research outcomes but treating the infection is not a viable alternative (Devan, 2011). Transmission of viruses through incoming materials including animals intended for experiments often becomes subclinical which has been a great concern to many researchers, veterinarians, managers and animal care staffs at laboratory animal facilities. The biological materials (cell lines, sera, sperm, and embryonic stem cells) may result in transmission of adventitious agents which can be ideally procured from defined vendors or required to screen them before use (Bhatt, 1986, Lipman 2000, Agca 2007) and inadvertent use of contaminated cell lines in immunocompromised mice can incubate and multiply in the hosts by conducive environment thereby infection can occur within the colony. It is important to prevent the spread of agents by surface disinfection of shipper boxes with adequate contact time (Mahabir 2008) otherwise, transmission can happen which further contaminate quarantine/holding animals (Reuter & Dyskyo 2003). However, there may be a potential risks for introducing any causative organisms (White 1998) even though adequate precautionary measures being taken care for animal shipments. The prevalence of pathogens and outbreak management varied among facilities, but these factors can predispose cross contamination in colonies (Carty, 2008). The Federation of European Laboratory Animal Science Association (FELASA) recommends screening of laboratory rodents and biological materials for a broad variety of agents and even if latent infection also can alter the experimental results through physiological changes and increases inter-individual variability which eventually higher number of animals used as a consequence to obtain valid results (Hofler, 2014). The recommendation for establishing of health monitoring programs has been emphasized as well as harmonization of global standards also suggested for laboratory animals (Nicklas, 1996, Weisbroth, 2000, Gaertner, 2007). In addition, FELASA recommends accreditation of diagnostic laboratories and health monitoring systems for breeding colonies and experimental units (Nicklas 2010). Similarly, Food and Drug Administration (FDA) also reiterated that monoclonal antibodies and other biotechnology derived products from tissues and/or cell lines originated from animals should be free of viral contamination prior to use either diagnostic or therapeutic purposes (FDA, 1998). Therefore, establishing a health monitoring program has been indispensable in breeding and experimental facilities (Mahler, 2014). Molecular methods are being employed commonly to detect rodent pathogens, these are aimed at detecting the nucleic acid (DNA or RNA) genome of etiologic agents (Ramachandra, 2016, Bauer, 2004) and multiplex PCR assay also developed as useful tool for monitoring rodent pathogens and viral contamination of biological materials (Wang, 2013). The advent of Multiplexed Fluorometric ImmunoAssay (MFIA) is a sensitive and specific serologic test designed to detect multiple viral and bacterial agents simultaneously in a single reaction well (Hsu, 2007) which offers high-throughput along with several advantages over the prevalent assays. Many traditional and molecular assays being used and its diagnostic features have been well documented (Compton, 2001). Generally, the diagnostic methods and agents selection is based on the institutions requirements as well as type/duration of experiments carried out in rodents. Overall, the challenges of importing animals from distant places with multiple transits dictates a need of incoming animals to undergo stipulated quarantine period followed by timely screening of pathogens prior to release for experiments. Considering the biosecurity and facility's need, a plan was proposed to establish in-house laboratory for timely analysis apart from the diagnostic services of outside laboratories.

Materials and Methods

1. Program Overview

Syngene Laboratory Animal Research (SLAR) facility at Syngene International Limited provides discovery and development support to various therapeutic areas of pre-clinical research. The facility has been importing rodents (transgenic, immunocompromised mice) and non-rodents (guinea pig, rabbits) from approved vendors (USA/Europe/India) on weekly basis in multiple shipments. Though, this facility procure animals from clean sources (barrier bred) and categorized as Class I (organisms; - / -); but at our facility, incoming animals are considered as class II (- / +); due to transportation (air and truck) associated with multiple transit which forced us to house the animals under stringent quarantine procedure (upto 21 days) followed by microbiological screening, parasitological examinations, environmental monitoring and routine clinical checks being performed apart from the established sentinel program.

2. Establishment of In-house Diagnostic Laboratory

Requirement of animal numbers have been increased over the years to cater several ongoing programs and so also import of special strains for therapeutic areas. The timelines and age of animals are important prior to release from quarantine

into the holding areas. Considering the above, samples (sera/tissue/blood and DBS cards impregnated with blood) have been shipped to various diagnostic laboratories and received results. Thereafter, a space was identified to set up in-house laboratory (LADDEL - Laboratory Animal Disease Diagnostics and Experimentation Laboratory) with clusters of 4 rooms in the same enclosure to perform diagnostic assays independently and also to prevent cross contamination, followed by serological and molecular diagnostic instruments were procured for installation.

3. Testing Strategies for Pathogens

The pathogens are categorized based on quarantine and/or sentinel monitoring frequency to screen the samples in-house (ELISA/IFA/PCR) as well as outside laboratories such as Bristol Myers Squibb (BMS), Wallingford, USA (MFIA/ELISA/IFA), Biodoc Germany (ELISA/IFA) and Vivo Bio Tech, India (PCR) as per the testing regimen framed for comprehensive health monitoring program. As part of our in-house requirement and to achieve timely third party testing, the above commercial laboratories were employed for periodical screening apart from in-house sample analysis.

3.1. Enzyme-Linked Immuno Sorbent Assay (ELISA)

The principle of indirect ELISA is to detect either antigen or antibody in the sera by two step binding process of primary and labeled secondary antibody by using commercial kits (XpressBio Life Science Products, USA). ELISA Reader and plate washer (BioRad iMark™) employed by following standard protocol as per the manufacturer's procedure manual. The reading was taken at 405 nm for the viral pathogens and results interpreted as difference (Δ) greater than or equal to 0.300 was considered as positive for the test.

3.2. Immunofluorescence Assay (IFA)

The indirect IFA used a primary antibody to recognize antigen and then a secondary fluorophore linked antibody which recognizes the bound primary antibody. The antigen preparation can be infected cells, a native cell line, tissue section, or recombinant antigen proteins. The XpressBio IFA test kits procured (Xpress bio life science, USA) and standard procedure adopted as per the instruction of manufacturer. The negative and positive control slides reading were taken at 100x magnification from a fluorescent microscope (Nikon with NIS-D software).

3.3. Polymerase Chain Reaction (PCR)

The integral PCR equipment's (Thermal Cycler 1000 with Real Time PCR-CFX96, Gel Doc™ XR+ and Electrophoresis chamber - BioRad) and Nano Drop (Thermo Fischer Scientific), were installed to amplify a single or few copies of a segment of DNA across several orders of the magnitude to generate multiple copies of a particular DNA sequence. The DNA and RNA extraction of blood and/or tissue were performed using commercially available veterinary PCR kits (BioinGentech, Chile/Minerva Biolabs, Germany). The PCR based diagnostic was used for early detection of selected pathogens during quarantine period in order to release the clean animals for experiments.

3.4. Multiplexed Fluorometric ImmunoAssay (MFIA)

The MFIA (Biorad Bioplex 200 System, USA) has been highly efficient in detecting several pathogens in single analyte rapidly and precisely. The Bio-Plex suspension array designed as unique 96-well fluorescent microplate reader and Bio-Plex Manager™ software was used for data analysis. The system can distinguish 100 different color coded polyesterine beads of each bearing unique homogenous capture assay using a flow based dual detector system which can simultaneously perform 100 different assays using 50µL of sample volume. The reagents were procured from Charles River Laboratories, USA and analyzed at Wallingford veterinary sciences diagnostic laboratory, BMS, USA. The sentinel and/or suspected samples were sent for MFIA assays apart from in-house screening for selected pathogens to ensure as backup system.

3.5. VITEK 2 Compact System

The identification of bacteria was established with VITEK 2™ Compact system USA, based on in-house requirements, the following reagent cards (GN - gram-negative fermenting and non-fermenting bacilli; GP - gram-positive cocci and non-spore-forming bacilli; YST - yeasts and yeast-like organisms; BCL - gram-positive spore-forming bacilli) were used for the identification of different organisms. The procedure was adopted from VITEK 2 compact standard protocol and exploration of organism's identification was the extent of contact plating method from swab samples taken from weekly incoming/sentinel animals. The standard culture (*Escherichia coli* - NCTC 12923*; Staphylococcus aureus*- NCTC 10788*; Bacillus subtilis*-NCTC 10400*; Candida albicans*-NCPF 3179*)* were used for validation and obtained from Biomerieux, India.

4. Environmental and parasitological examinations.

The comprehensive microbial monitoring includes animal rooms along with ancillary areas of vivarium. However,

ATP bioluminescence test was carried out on weekly basis followed by contact plates on monthly intervals to assess the microbial load in the animal rooms as part of sanitization procedure. As a standard practice, swab samples from weekly animal shipments (throat, rectal) as well as sentinels (trachea and caecum) were used for contact plates. Generally, if more growth enumerated as averaged (> 50 cfu/room or animal) which can determine whether to identify the particular organisms and/or decontamination of that particular enclosure in order to minimize the spread of contamination. In addition, tape testing and fecal floatation technique were performed routinely from sentinels which includes pathological screening as and when needed as part of extended diagnosis.

Results

The diagnostic instruments were procured in parallel with laboratory modification and then installed followed by trainings provided to staffs. Subsequently, validation was performed by using commercial kits for pathogens based on limited/full panel testing requirements. The ELISA was established as primary assay followed by IFA, PCR and VITEK for identification of microorganisms. The validation test kits of positive controls procured were demonstrated by IFA method (Figure 1). Similarly, PCR validation results showed that consistent with commercial kits for initially selected organisms (Sendai virus, *Mycoplasma pulmonis*, Mouse hepatitis virus, Lymphocytic Choriomenintgitis virus, Rotavirus, Parvovirus) and rest organisms were ensured subsequently (Table 1). However, the validation results of standard culture of bacteria and fungi (*Escherichia coli; Staphylococcus aureus; Bacillus subtilis; Candida albicans*) showed 96-99% probability of at least 60 biochemical parameters analyzed by Vitek system and confidence criteria showed as very good to excellent. The results of PCR, MFIA, ELISA and IFA were monitored for any test positives to understand the health status of animals and further reconfirmation system was also established. In addition, ATP bioluminescence and contact plates (data not shown) results were evaluated to determine the decontamination process and/or further identification of organisms by Vitek as part of exploration.

Discussion

The in-house diagnostic laboratory set up has played a pivotal role for timely screening of agents and early results which has been a prerequisite to determine the quarantine release. The in-house diagnostics including commercial laboratories screening provided a scope to analyze samples by various methods. The previous report suggested that a risk-based allocation of health-monitoring resources should concentrate frequency and/or sample size on these high-risk agents, and monitor less frequently for the remaining, lower-risk, infectious agents (Pritchett-Corning 2009). A report described that the types of diagnostic tests available, which agents deserve monitoring, and the appropriate frequency for such interventions (Livingston 2003) needs to be established. At our facility, representative samples (2-5%) of incoming animals have been randomly tested at different intervals by adopted methods such as PCR (5-7days); ELISA/IFA (14-21 days); including throat, rectal swab for culture plate as well as parasitological examinations. Similarly, MFIA (6, 12 & 18 weeks) screening was performed along with in-house ELISA followed by parasitological examinations (tape test/floatation technique) in order to ensure health status of colonies housed within the facility. The PCR assay provides rapid results with sensitivity and specificity of agents because these are aimed to detect nucleic acids of any particular pathogenic organisms. A report investigation revealed that the PCR assays were used to identify rat parvovirus 1 (RPV-1) and rat minute virus 1 (RMV-1) from the naturally infected rats and these assays were shown as sensitive, specific for the RPV-1 and RMV-1 in rats (Wan 2006). Similarly, mouse parvo virus (MPV) inoculated in different strains with two different ages were evaluated for virus-specific DNA or antibodies detection (4 weeks post inoculation) and opined that PCR analysis and serologic assays should be considered in the context of age with strains exposed especially for sentinel monitoring (Besselson 2000). Moreover, an earlier study reported that fluorogenic nuclease PCR assays provided a potentially high-throughput to detect rodent parvoviruses in infected mice and contaminated biological materials (Redig 2001). On the other hand, an assay was developed for antemortem PCR to detect mouse parvovirus-1 (MPV) and mice minute virus (MMV) using faecal samples (1-7 days held at room temperature) after the infection shown positive for MMV and MNV. In addition, fecal shedding was detected for MMV in 60-70% of mice (5-7 weeks old) and shown negative by 13 weeks; thereafter, MPV fecal shedding in 90-100% of mice was noted (5-11 weeks) followed by disappeared and further suggested that 5-9 weeks old mice can be ideal to detect MMV and MPY by PCR method (Bauer 2006). However, a comparison of PCR and MAP testing methods reported that the results of PCR becomes available within 2 days, while comparing with MAP testing which has shown almost 28 days to complete the test (Blank 2004). Additionally, a random-source DNA samples obtained from naturally infected laboratory mice (n=381) were evaluated by PCR and RFLP analysis to determine the prevalence of murine parvovirus strains circulating in contemporary laboratory mouse colonies (Besselsen *et al* 2006).

The ELISA has been commonly used as serologic test to detect antibody produced against the infectious agent and highly sensitive, rapid and relatively inexpensive. The mouse parvovirus was validated for its sensitivity and serogroup-specific diagnostic test, the results revealed that higher sensitivity (93.3% vs 65%) of MPV ELISA than the specificity (98.3% vs 99.3%) to rNS1 ELISA and suggested that MPV VP2 ELISA provides a sensitive and

serogroup-specific alternative for diagnosis in mice (Ball-Goodrich 2002). Nevertheless, one can use other tests of at least equivalent sensitivity and reliability, including PCR tests provided that adequate validation of each methods should be performed with respect to sensitivity, selectivity (specificity), reproducibility, and stability of such validation experiments must take place on validated equipment's with quality-controlled process (Blank 2004). The advancement of high-throughput diagnostics and rapid results provides advantages in terms of sample volume and turnaround time, hence, alternative methods can also be adopted for a range of assays to become comprehensive for early detection of agents based on its sensitivity, selectivity and limits of detection as the nature of causative agents varied among rodent colonies due to many factors such as age, strain, mode of transmission, type of containment, husbandry practices etc. The methods adopted should be validated by quality control standards at in-house along with documentation of results for consistency and reliable data which eventually provides traceability in future. Moreover, it was suggested that repeated testing of biological materials may not be necessary if the records have been maintained meticulously (Peterson 2008).

At our facility, animals and biological materials have been screened before proceeding into *in vivo* experimentation from vivarium standpoints as well as research integrity. Sometimes, the assays performed may overlap with other methods and considered that multiple animal consignments from barriers with different ages of several strains procured which have been considered unknown health status while on receipt at quarantine. Although, clinical manifestations and shedding of organisms can be varied among age groups/strains and other factors associated for diminishing the infections. The comprehensive screening methods of selected organisms at periodical interval provide confidence on animal health as well as established quality standards being practiced at laboratory animal facility. In cases, positive/suspected results obtained were tested in a programmed manner i.e. retest the sample or other methods; retest at different laboratories; correspondingly resampling from the same colony to repeat these tests; suspected animals can be contained by appropriate methods. The confirmed and/or unexpected positive results if any should be discussed at site by veterinarians/investigators/ stakeholders to decide further apart from vendor's health monitoring results also verified on routine basis with respect to the consignments received from the stipulated periods.

Serological assay false positive may be due to incomplete antibody and nonspecific cross-reactive antibody. Similarly, PCR false positive can be ruled out by other samples, sequence amplified for previous test run of same target gene and positive controls. In spite of the above, serosurveillance of most samples were screened by ELISA as confirmatory due to its sensitivity and PCR based diagnostic was used for early detection of selected pathogens during the quarantine period in order to release the clean animals for experiments. The IFA method has been used as confirmatory method for ELISA/MFIA/PCR in cases where false positives and/or suspected samples if any. Additionally, some of the animals were screened at vendor site based on request before the animal shipment dispatch to SLAR. As part of the organization's business continuity plan as well as to obtain third party laboratory testing, the above commercial laboratories were employed strategically for periodical screening apart from in-house testing which collectively upkeep the health standards of animals maintained and used for research. Nevertheless, proactive risk assessment of multiple shipments during receipt and release of clean animals for experiments have become labor intensive in spite of substantial costs and time involved. The high-throughput diagnostics as well as programmed testing has been reduced substantial turnaround time of results, irrespective of number of samples being analyzed with backup methods in different laboratories which served as historical data. The entire process has been systematically ensured to perform the activities by considering animals are being our central focus of drug discovery research and provided assurance to animal resource program personnel working in vivarium facilities.

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Table 1. List of organisms screened by various diagnostic methods during quarantine and sentinel monitoring program.

List of Organisms	Assay type	Species
Parvovirus (MPV1 & MPV2)	ELISA/MFIA/PCR	M
Mouse Hepatitis Virus (MHV)	ELISA/MFIA/PCR	M
Minute Virus of Mouse (MVM)	ELISA/MFIA	M
Pneumonia Virus of Mouse (PVM)	ELISA/MFIA/PCR	M
Lymphocytic Choriomeningitis Virus	ELISA/MFIA	M
Epizootic Diarrhea of Infant Mice (EDIM)	ELISA/MFIA/PCR	M
Mouse NoroVirus (MNV)	ELISA/MFIA	M
Ectromelia Virus/Mouse pox	ELISA/MFIA	M
Lactate dehydrogenase-elevating virus	ELISA/MFIA/PCR	M
Sendai Virus	ELISA/MFIA/PCR	M, R
Reo Virus type 3 (REO-3)	ELISA/MFIA	M, R
Adenovirus (FL/K87)	ELISA/MFIA/PCR	M, R
Theiler's Murine Encephalomyelitis Virus (TMEV)	ELISA/MFIA/PCR	M, R
Mycoplasma pulmonis	ELISA/MFIA/PCR	M, R
Corona Virus	ELISA/MFIA/PCR	R
Pneumonia Virus (PVR)	ELISA/MFIA	R
Parvovirus (RPV)	ELISA/MFIA	R
Hanta Virus	ELISA/MFIA	R
Kilham/H1 virus	ELISA/MFIA/PCR	R

Note - M - Mice; R- Rats; IFA tests performed in case of suspected/positive by ELISA. Sample types - ELISA (Serum); MFIA (Serum/Blood in DBS cards); IFA (Serum) tested as confirmatory for most organisms; PCR (Blood/Tissues/Lymph nodes); Parasitological screening methods not shown.

Figure 1: Parvovirus positive control image (100x) shown by IFA method

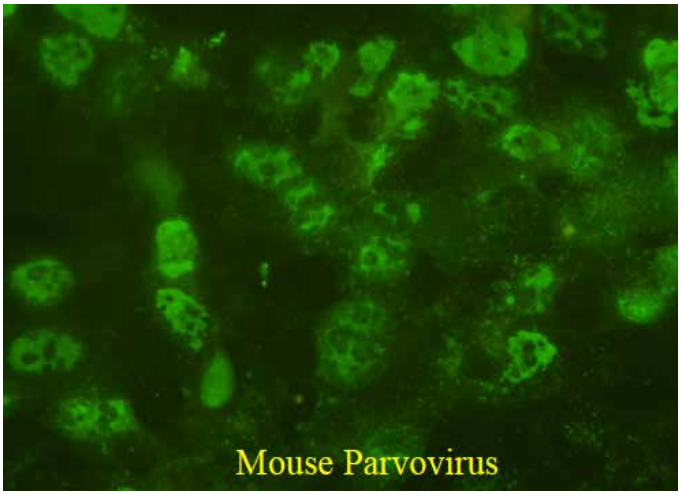

Figure 2: Gel Doc image captured from Mouse Hepatitis Virus (MHV)
(T1-T4 test samples; IC Internal Control; PC- Positive Control; NC-Negative Control).

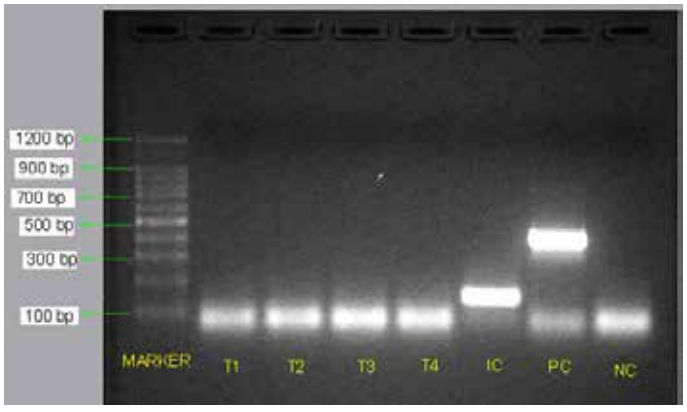

Figure 3: Gel Doc image captured from *Mycoplasma pulmonis* analysis
(NC-Negative control; PC-Positive Control; T1 - T4 test samples).