Effectiveness of Sanitization Assessment by ATP Bioluminescence Detection System as a Complementary Method in Vivarium: A five-year status report

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Abstract:
Sanitization is an important activity performed in the laboratory animal facilities and periodical assessment of sanitization provides confidence in surface cleanliness as well as health status of animals in the vivarium. The primary objective was to evaluate routine disinfection and/or sanitization practices upon verifying the adenosine triphosphate (ATP) bioluminescence method, which further expressed as relative light units (RLU), a relatively easy and rapid method to interpret results within a minute after the swab sampling performed on any surface. A five-year data compilation showed that RLU values were within the in-house acceptable limits of animal rooms sampled from racks, isolators, doors, trolleys, cage changing stations, tables, walls and cage accessories. However, some of the materials such as racks and trolleys of high-traffic areas showed a significant increase in RLU values due to organic matters that might be present on the equipment surfaces but recorded values were well within the limits set by the facility. Additionally, contact plates were also used as confirmatory method to evaluate microbial monitoring in animal rooms including cage accessories and further historical values of RLU provided confidence to increase monthly contact plate sampling interval to a quarterly basis and followed as per the schedule. Moreover, representative samples from incoming animals were screened by microbial monitoring at regular intervals during the quarantine period and active sentinel samples also screened for serology or PCR as part of comprehensive health monitoring program. In conclusion, ATP method can be used to assess the real-time effectiveness of sanitization practices in vivarium as it provides immediate feedback to animal care personnel that enables corrective actions; hence the ATP bioluminescence is continued as one of the complementary methods at our laboratory animal facility.

Keywords *- Adenosine triphosphate (ATP), Bioluminescence, Luminometer, Rapid microbiology, Relative Light Units (RLU), Vivarium Sanitization.*

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Introduction

Monitoring of sanitization is an essential activity in laboratory animal facilities (Turner *et al*, 2010), and maintaining cleanliness in vivarium has always been an imperative step to avoid any potential cross-contamination which in turn helps to maintain disease-free animals as well as the clean status of the facility (Devan *et al*, 2011). The sanitary environment has been a well-recognized principle in animal facilities and several methods are being used for microbiological monitoring of environmental surfaces, which include swab rinse, sponge rinse, direct rinse, contact plates and replicate organism detection and counting (RODAC) plates. The contact plates are easy to use and require an incubation period (48h) before obtaining the results of microbial counts. However, contact plates may not provide immediate results about disinfection or sanitization for items such as racks, cages, animal rooms and surgery suites including equipment or any inanimate objects in case of unacceptable results are obtained. The adenosine triphosphate (ATP) bioluminescence, a well-established technology, ubiquitous molecule that mainly acts as a major energy carrier for all living cells (plants and animals) including microorganisms is being used. Over the years, rapid microbiology using the ATP luciferase technique has been applied as a convenient methodology for the global enumeration of microorganisms in a wide range of samples (Stanley, 1989) including the improvement of cleaning processes (Branch-Elliman *et al*, 2014), laboratory animal facilities (Capria *et al* 2022; Allen *et al* 2021; Turner *et al*, 2010; Edine *et al*, 1998; Schondelmeyer *et al*, 2006, Horn *et al*, 2012) including cage washers in the vivarium (Walker *et al*, 2021), hospitals (Willis *et al*, 2007; Lewis *et al*, 2008;

Ayclcek *et al*, 2006) especially operating theatres (Sanna *et al*, 2018), dairy and food industries (Stanley *et al*, 1989; Lappalainen *et al* 2000, Carrascosa *et al*, 2012; Vilar *et al*, 2008).

The rapid ATP testing offers a real-time indication of cellular contaminants of the surface or device cleanliness (Pontes *et al*, 2023). The ATP testing provides a quick, convenient method to assess cleanliness (Bruno-Murtha *et al*, 2014) and this assay can set up a linear relationship between ATP concentration which corresponds to light output, and measurement of results in a sensitive luminometer expressed as Relative Light Units (RLU). However, ATP bioluminescence provides accurate and valuable data regarding washer/disinfectant efficacy, contributing to quality control mechanisms (Heathcote *et al*, 2009). In general, visual assessment has been employed to judge surface cleanliness and is considered subjective which becomes relatively insensitive. Although, ATP swabbing results are not directly equivalent to microbial monitoring but can provide a good indication (Willis *et al*, 2007) of whether the surface is clean by indicating the degree of contamination rather than definitive microbial numbers (Colquhoun *et al*, 1998) and offer instant feedback to the facility personnel on surface cleanliness and deficiencies in cleaning protocols adopted by the institution (Lewis *et al*, 2008). Moreover, ATP testing offers greater benefits over culture methods and its ability to produce rapid results enabling immediate corrective action if any unacceptable RLU values are observed in the animal rooms. Considering the above, the ATP method was introduced to evaluate the random sampling assessments to understand the biweekly status at specified locations of animal rooms including cage accessories apart from monthly contact plates sampling along with other microbiological monitoring in vivarium.

Material and Methods

Facility Overview

The effectiveness of sanitization was assessed by the ATP bioluminescence method at Syngene International Limited (AAALAC accredited). ATP sampling was performed in all the areas viz, a). Clean area (sterile store, unloading area of autoclaves and cage washers

b). Animal holding area (quarantine, breeding, oncology, experimental rooms, laboratories, surgery and recovery suite. c). Unclean area (service corridors, quarantine receipt area, waste disposal including necropsy). The facility construction material is prefabricated ISO class 8 (class 100,000) clean room panels with barrier maintained 100% fresh air supplied from the ceiling and return raisers at the bottom of rooms integrated with HVAC units providing 15-20 Air Changes Per Hour (ACPH) and High Efficiency Particulate Air (HEPA) filters integrity is validated annually by poly-alfa-olefin (PAO) or replaced as and when required. The HVAC units are integrated with the Building Automation System (BAS 24x7) and provide the status of at least 30 HVAC units as part of barrier maintenance of the facility. The joint-less epoxy floor finish with coving and fenders also installed to protect the wall panels in animal rooms and corridors. Moreover, the facility is also equipped with two mechanical cage rack washers and two walk-through autoclaves as redundant backups to ensure the clean supply of materials and cage accessories for routine husbandry in vivarium.

Bioluminescence Method

The advancement of rapid microorganisms' detection and ATP bioluminescence-based luciferin / luciferase reaction has shown great interest in various fields. The detection of ATP through ATP-luminescence technology is a method of choice to replace traditional methods and reduce time to produce results without losing reliability. Hence, the enhanced sensitivity of pocket swabs was used by the novaLUM luminometer (Charm Sciences) in the strategic locations to measure ATP not only from the room level microorganisms but also from animal house accessories. The observed values expressed as RLU and swab samples were strategically collected from the following location such as racks, isolators, doors, cage changing stations, trolleys, walls, tables and cage accessories in animal rooms.

Animal Husbandry Practices

The routine cleaning and/or mopping activities were performed twice daily and ATP sampling was collected at biweekly intervals for a period of five years. Animal husbandry and care activities of the planned rooms / areas were closely monitored so that sampling performed without any interference with routine husbandry and/or experimental activities. Laboratory animals are housed in autoclaved bedding materials (corncob / arbocel) with enrichments and maintained using individually ventilated cages (IVC) / Digital ventilated cages (DVC) / Isolators from quarantine to experiments. The cage changing frequency was followed as per the facility standards and non-rodents were housed in a standard rack system with enrichments.

Sampling Procedure and Estimation

The ATP samples were collected from visibly clean surfaces, if any visible soiling or residue was apparently noticed on the sampling surface were re-cleaned before collecting samples or sampled on the next day. The pocket swab device was designed by manufacturers in such a way that it can be removed by rotating the cap from the package and the swab tip comes pre-moistened with a detergent that breaks down biofilm on the test surfaces. Adequate care was taken while sampling to avoid touching the swab tip or shafts by fingers to prevent contamination, if any, which in turn may show false RLU values. The samples were collected on regular and/or irregular surfaces in a square (4 x 4 inch) area of each identified locations, thereafter, the device was held upright activating the pocket swab gently shaking (side-to-side motion) and bathing the swab bud into the stable liquid reagent to activate bioluminescence reaction and reading was taken within 5 to 60 seconds of its activation. In general, effectively cleaned areas can show low ATP concentrations and less light produced which in turn appears low RLU reading indicating less contamination and vice versa. The ATP sampling was carried out at biweekly intervals to assess the effectiveness of cleaning procedure and subsequent contact plate sampling was also performed on a monthly rotational basis so that all the animal rooms/areas are covered within each quarter. Considering the physical facilities and complexity of traffic patterns, the animal rooms

were divided into three units (I, II, III) based on the feasibility and further synchronized with husbandry activities prior to sampling (4-5 locations per room).

Statistical Analysis

All the quantitative data presented as mean ± SEM and a value of $p \leq 0.05$ was considered statistically significant. The statistical tests were applied using Graph Pad Prism Software Version 5.0, USA.

Results

The ATP sampling of biweekly RLU data was compiled and represented on a monthly basis (n=60) for a period of five years. The instrument was calibrated on a quarterly basis using positive control tablets (33756 ± 5149) as well as negative control tablets which showed 0 at all-time points and ensured the sensitivity of the luminometer (recommended by the manufacturer as positive control values between 10000 to 40000 RLU). The RLU values were validated initially across all the animal rooms including corridors and procedure areas to set the limits. During the standardization phase, some of the values of the area appeared higher than 20000 RLU (data not shown) and the subsequent cleaning/mopping procedure was refined with respect to quarterly disinfectant agents change and adequate contact time to minimize the contaminants in order to bring down the RLU values. The animal room (AR 29) and necropsy room (AR 63) showed a significant increase in RLU values when compared with other rooms **(Figure 2 & 3**). Similarly, a significant increase of RLU showed at quarantine suite racks / isolators surfaces (2423.4 ± 52.6) and trolleys / cage changing stations (2334 ± 76.6) (**Figure 6)** as well as breeding suite showed significant increase of RLU values from trolleys (2833.9 ± 196.1) (**Figure 7**). Moreover, the compiled colony forming units of RODAC plates observed initially were high (1.71 ± 1.08) (**Figure 9)** in comparison with subsequent annual summary data. On the other hand, there was no significant differences shown in animal room unit I (**Figure 1**), husbandry corridors (**Figure 4)**; clean store (**Figure 5),** and oncology suite (**Figure 8**) when compared with the corresponding areas within the same rooms/locations.

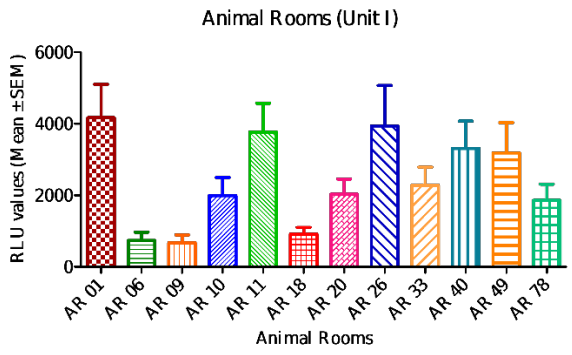

Figure 1: Animal Rooms (Unit I) of RLU values (n=60; Mean ± SEM) from 4 different locations. One-way ANOVA

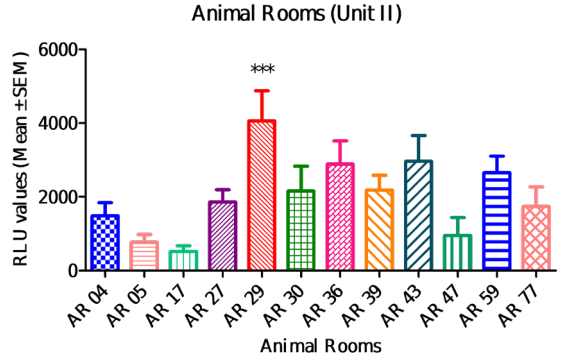

Figure 2: Animal Rooms (Unit II) of RLU values (n=60; Mean ± SEM) from 4 different locations. One-way ANOVA (*** P<0.001) *Indicate significant differences among sampling points.

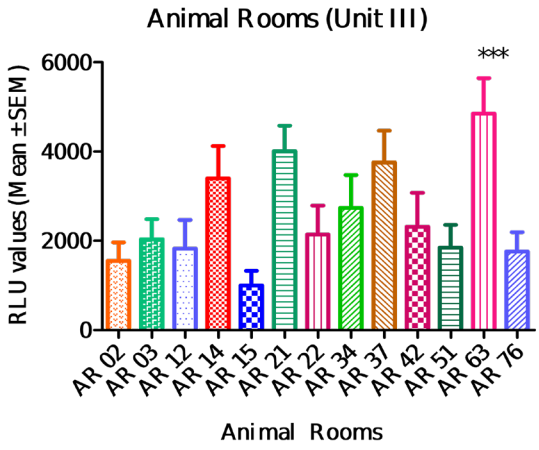

Figure 3: Animal Rooms (Unit III) of RLU values (n=60; Mean ± SEM) from 4 different locations. One-way ANOVA (*** P<0.001) *Indicate significant differences among sampling points.

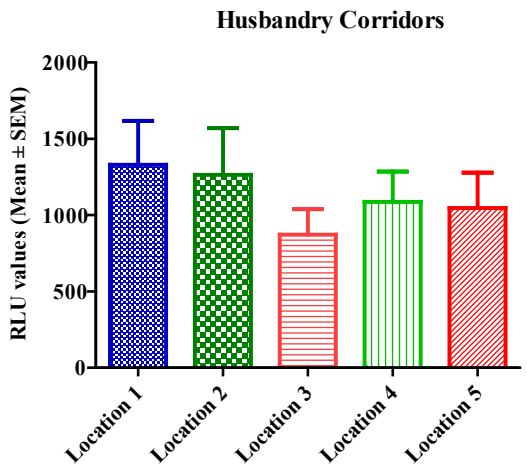

Figure 4: Husbandry corridors RLU values (n=60; Mean ± SEM) from 5 different locations.

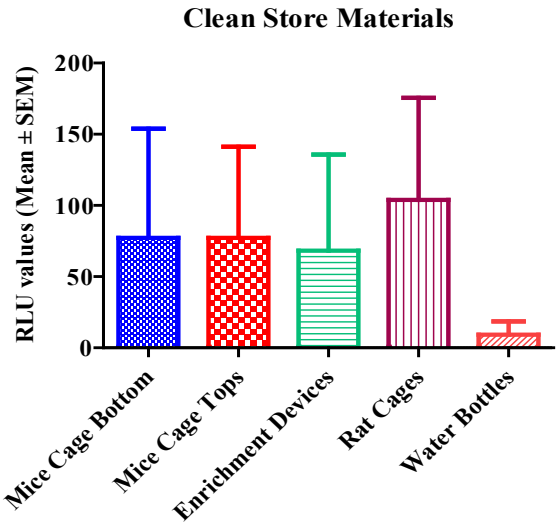

Figure 5: Clean store materials RLU values (n=60; Mean ± SEM) from 5 different autoclaved materials stored for husbandry supplies.

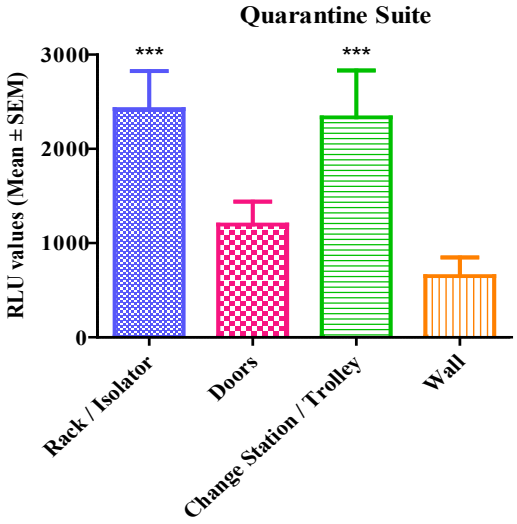

Figure 6: Quarantine Suite RLU values (n=60; Mean ± SEM) from 4 different areas. One-way ANOVA (*** P<0.001) *Indicate significant differences among sampling points.

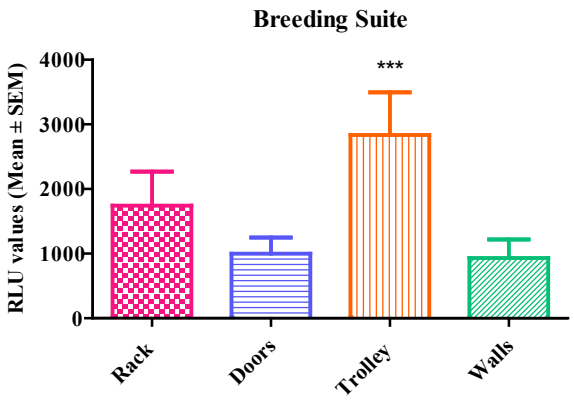

Figure 7: Breeding Suite RLU values (n=36; Mean ± SEM) from 5 different areas. One-way ANOVA (*** P<0.001) *Indicate significant differences among sampling points.

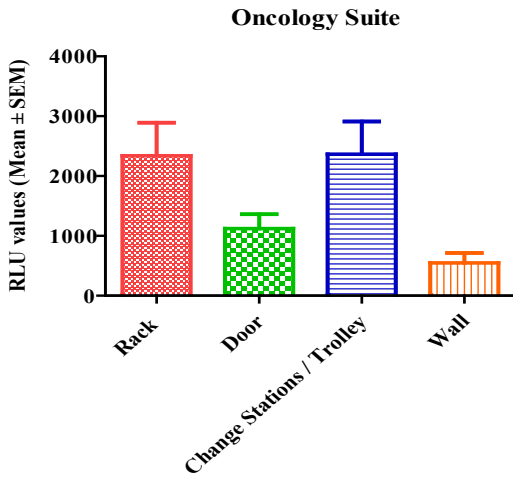

Figure 8: Oncology suite RLU values (n=60; Mean ± SEM) from 4 different areas.

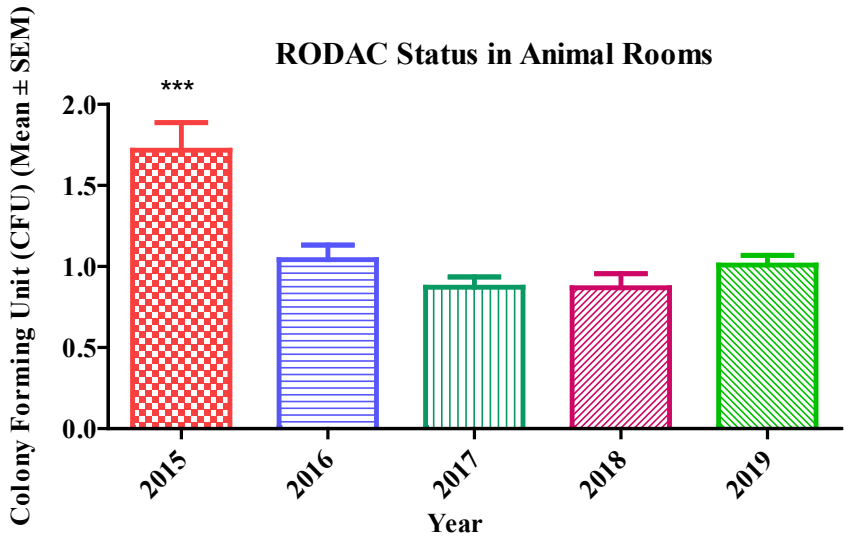

Figure 9: RODAC plates values of monthly random sampling was taken from animal rooms, corridors and represented for 5 years. One-way ANOVA (*** P<0.001). * Indicate significant differences among sampling points.

Discussion

The ATP bioluminescence RLU values provided quantitative data that indicates the effectiveness of cleanliness in the animal rooms. Critical areas such as quarantine, breeding, and oncology were monitored by access control to avoid any cross-contamination, and the scientist corridor (clean corridor) as well as service corridor (dual corridor), traffic pattern was always ensured to minimize the contamination across the barriers. Generally, service corridor operation was monitored in a timely manner as per the set protocol and ensured mopping before and after each clean and unclean activity. However, a significant increase in the ATP values of trolleys/cage changing stations observed from quarantine, oncology and breeding areas was due to irregular surface finish of trolleys (made up of plastic) or frequent usage of cage changing stations where animals routinely transferred. Similarly, racks/isolators surfaces showed increase in RLU values at quarantine and oncology suites, possibly due to the high inflow of weekly quarantine animals and the high-density animal population of this particular suite. Based on the in-house validation, ATP values below 10000 RLU were set as an acceptable limit and considered further to use materials subject to the kind of activities carried out. The previous study reported that the bioluminescence monitoring device was found to be more efficient and significantly less expensive than RODAC plates as the sanitation monitoring tests were performed on the animal room floor after cleaning by a variety of methods and on the cages washed in mechanical cage washers (Edine *et al*, 1998). In general, the vivarium had a variety of functions with active experiments which led to the presence of organic matter even though cleaning/mopping was performed twice daily or as and when required. A hypothesis was tested on caging accessories for organic contamination using ATP measurement (luciferase test swabs) as well as bacterial CFU (RODAC plating) which showed no significant differences in ATP values (14 - 180 days) and bacterial counts (up to 120 days), thereafter significant increase was observed in gram-negative bacterial contamination (90 - 180 days) which was below the allowed limits of American Public Health Association (APHA) and further concluded as clinically insignificant including the biological context (Schondelmeyer *et al*, 2006). Similarly, another study was conducted in rats and mice using three different bedding materials (aspen, cellulose and aspen:cellulose) and ATP concentrations were measured that appeared lower in the cages maintained rats with aspen:cellulose bedding than aspen or cellulose alone. However, mice cages showed higher ATP levels with aspen (6 weeks) and observed that ATP concentrations were increased between 2 and 6 weeks and appeared to reach a maximum threshold of around four weeks in both species (Horn *et al*, 2012). A study was conducted in a rodent facility with wire bar lid inserts and cage tops at different intervals using the ATP method from the static and IVC mice and rats cage accessories and determined that the actionable level above 100,000 RLU of all the groups evaluated (Allen, 2021). Similarly, another study was conducted using mouse cage components ranging from 4-, 6- and 8-weeks period when compared with 2-weeks, the results suggested that the sanitation frequency can be increased to 6 weeks based on the performance standards (Ball 2018).

As part of routine health monitoring surveillance, all the incoming animals were housed in quarantine (up to 21 days) and samples were collected at different intervals, as per the set procedure for microbial analysis, serology (ELISA/MFIA), and PCR analysis apart from active sentinel program in place. The instances where higher RLU values indicate the hygiene status of any specific room provided scope for husbandry staff to determine whether to repeat the sanitization procedure. Thereafter, persistent higher values warranted interim decontamination apart from the routine cycles performed every two months intervals using either vaporized hydrogen peroxide, chlorine dioxide or Virkon-S. Moreover, it was

experimentally demonstrated that serial dilutions of *Escherichia coli*, *Staphylococcus aureus*, *Toxoplasma gondii* Tachyzoites, *Toxocara canis* eggs, epithelial cells, rodent blood, urine and faeces showed that ATP method was showed a strong degree of linear predictability which sensitively detects pure cells as well as organic contaminants, but the limitation was poor detection of gram-negative bacteria due to its incomplete cell lysis (Turner *et al*, 2010). The previous study emphasized that the ATP or microbiologic evaluation was a better method to assess the cleaning efficacy and reported by the ATP method (26000 RLU) correlated with RODAC (10 CFU/plate) on floor sanitation (Malik *et al*, 2003). However, our results corroborated with earlier findings as five years of compiled data revealed (4848.9 ± 157.7) less than acceptable range of the facility set values (<10000 RLU) as well as RODAC compiled values also showed (< 2 CFU) within the facility accepted range (up to 50 cfu in any non-decontaminated areas) because the sampling was performed on random days by the trained staff. Considering the above results, we have extended the usage by optimizing the validity of autoclaved materials until 7 days for cages with beddings, water bottles and 14 days for cage tops, enrichments and the racks housing interval also extended up to 90 days. In case of any materials are left unused for the stipulated period, then the material must be re-autoclave prior to the next use with periodical monitoring in the facility. However, the animal holding room showed a significant increase in RLU values (AR 29) because multiple racks were always placed with a large turnover of mice colonies for short-term experiments that could have attributed to increased RLU values. Similarly, the increased RLU values of the necropsy room (AR 63) also interpreted as extensive use of this area or performing prosection as well as handling body fluids leading to the persistence of biological matters. A report of multivariate evaluation of operating rooms revealed that microbial counts declined over the period, but observed that ATP remains higher after the scheduled operations (Saito *et al*, 2015). Nevertheless, the surgery and recovery suite including clean store areas appeared to have lesser RLU values (103.9 ± 41.69) due to impeccable cleaning with limited usage and/or frequent decontamination process. In addition, the RLU (ATP method) levels were measured in zebrafish tanks, lids, and nets by comparing two disinfectants before and after the process showed a reduction of RLU values (96.6%) as compared to daily water replacement (91.2%), which further suggested that soaking for short duration (30 min) resulted in higher reduction (99.7%) of RLU in tanks and lids than longer duration (60 min) that has led to slightly lower reduction (97.1%) (Garcia *et al*, 2011). On the other hand, a laboratory investigation of *Bacillus anthracis* showed a significant correlation of ATP when compared with culture methods for vegetative cells except for spore form (Gibbs *et al*, 2014). However, a previous study reported a positive linear relationship (10^3-10^7) between ATP and aerobic plate count (APC) of *Lactobacillus rhamnosus* (Leon *et al*, 2007) and another experiment revealed that correlation existed between ATP and conventional plates of bacterial CFU's (10^3-10^8) in food samples (Luo *et al*, 2009).

Conversely, a study finding of health care systems under laboratory conditions reported that evaluation of *Staphylococcus aureus* with 4 different ATP detection systems (ensured its linearity and repeatability prior to use) for at least 14 disinfectants where the inoculum was directly applied over the swabs resulted significant differences in their sensitivity to detect viable microbial contamination on experimental surfaces. The results further suggested that most disinfectants were quenched and interfered with ATP readings (Omidbakhsh *et al*, 2014). The other reports also suggested that disinfectants were able to confound the ATP measurements (Lippalainen *et al*, 2000 and Mulvey *et al*, 2011). In dairy industry, the ATP bioluminescence method showed that 100 % inadequate hygiene on six stainless steel equipment surfaces and subsequently verified with culture methods resulted in 50% (APHA) and 33% (WHO) which suggested that high RLU variations observed on

surface analysis and was not correlated with mesophilic aerobic counts (CFU) of culture plates (Costa *et al*, 2006). Considering the above diverse reported findings, testing for microorganisms with culture method and enumeration of colony provide accurate information which involves laboratory with high skill levels for better outcomes. Hence, the ATP method may not be a replacement over culture methods but can be used as a complementary technique (Willis *et al*, 2007; Ayclcek *et al*, 2006; Carrascosa *et al*, 2012; Omidbakhsh *et al*, 2014) for rapid analysis and fast response to understand the sanitization status apart from established microbial methods for a greater comprehension of surface cleanliness in laboratory animal facility. Hence, further studies are recommended with commonly prevalent microorganisms of laboratory animal facilities as well as rodent and/or non-rodent pathogens to elucidate the extent of ATP detection in various contexts.

In conclusion, a compilation of five years of ATP results from various locations collected and analysed was well within the in-house acceptable limits of animal rooms. Therefore, a combination of both ATP bioluminescence as well as culture plate (RODAC) method was continued at periodical intervals which further provided better confidence on quality assurance program for a greater comprehension of surface cleanliness and sanitization practices in the vivarium facilities.

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Conflicts of interest

The authors declare No conflicts of Interest

Author Contributions

The authors contributed at various phases of the experiment such as designing, execution, sampling and data interpretation.

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