https://doi.org/10.31925/farmacia.2019.6.11 **ORIGINAL ARTICLE**

THE ATP BIOLUMINESCENCE METHOD: AN ALTERNATIVE APPROACH FOR MONITORING CLEANLINESS IN HOSPITAL PHARMACY CLEANROOMS

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Abstract

The objective of this study was to evaluate the feasibility of a new rapid adenosine triphosphate (ATP) bioluminescence method for monitoring surface hygiene in hospital pharmacy cleanrooms. It was assumed that, despite the low microbial load, the rapid method is sufficiently sensitive for routine use, and, due to its simplicity, useful as a rapid and effective tool for quality control, and also as a tool for detecting change and monitoring trends. A total of 537 surfaces in separate locations were randomly sampled with the traditional Replicate Organism Direct Area Contact (RODAC) imprint method (n = 484) to detect colony form units parallel to LuciPac Pen swabs (n = 992) to detect ATP load. The results were compared to assess correlation and to confirm the usefulness and limitations of the bioluminescent method. The statistical analyses were performed using McNemar's test, and Cohen's kappa coefficients of agreement were calculated for each room category. Defined cleanliness thresholds for LAF cabinets were 70 relative high units (RLU), for cleanrooms 140 RLU, and for filters 340 RLU. The results for the bioluminescent method largely confirm the data obtained with the standard culture method, and there was no significant difference in ability to predict unclean surfaces. However, despite the agreement of the results obtained, the analysis did not reveal a correlation between them. We concluded that bioluminescence can be useful as a supplementary method as a real-time surface hygiene monitoring tool to assess a room immediately before use. Faster access to data is an exceptional contribution to the quality and effectiveness of the process, as well as to product and patient safety.

Rezumat

Obiectivul acestui studiu a fost de a evalua fezabilitatea unei noi metode bioluminescente bazată pe adenozin trifosfat (ATP) pentru monitorizarea igienei suprafețelor din incintele curate ale farmaciei de spital. În ciuda încărcăturii microbiene scăzute, această metodă rapidă este suficient de sensibilă pentru a utilizarea ei în controlul calității, precum și ca instrument de estimare a tendințelor. 537 suprafețe din locații diferite au fost analizate prin metoda RODAC (*Replicate Organism Direct Area Contact*) (n = 484) pentru detectarea unităților formatoare de colonii in comparație cu metoda bazată pe ATP (*Pen*-urile LuciPac) (n = 992). Analizele statistice au fost efectuate folosind testul McNemar, iar coeficienții Cohen au fost calculați pentru fiecare categorie de cameră. Pragurile de curățenie definite pentru incintele cu flux laminar de aer au fost de 70 de unități relative, pentru camere curate 140 unități și pentru filtre 340 unități. Rezultatele metodei bioluminiscente sunt comparabile cu cele obținute cu metoda standard și nu există diferențe semnificative în capacitatea de estimare a gradului de încărcare microbiană a suprafețelor. În concluzie metoda bioluminescentă poate fi folosită ca instrument de monitorizare a igienei suprafețelor în timp real pentru a evalua o cameră înainte de utilizare.

Keywords: Cleanroom, bioluminescence, adenosine triphosphate (ATP), colony-forming unit (CFU), relative light unit (RLU)

Introduction

Traditional microbiological methods, which are the most commonly used methods for evaluating cleanliness, are neither optimal nor acceptable for routine monitoring of the hospital pharmacy production environment. They require sample cultivation and isolate identification, which are time-consuming, are expensive and do not provide immediate results [1, 2].

In recent years, rapid alternative methods have been developed, including bioluminescent assays based on measuring adenosine triphosphate (ATP) present on the surface observed. The ATP-bioluminescent method utilises a firefly luciferase-luciferin system, which catalyses the transformation reaction from ATP to adenosine monophosphate, resulting in light emission. The released light can then be measured with a luminometer, which produces a result expressed in Relative

Light Units (RLUs). The intensity of light emitted is proportional to the initial amount of ATP in the sample [3].

The bioluminescence method is easy to use and provides results in a few minutes. The method, which is already applied in food industries, healthcare settings and pharmaceutical industries, mostly measure overall cellular ATP and not only microbial ATP. Therefore, this rapid ATP testing is in fact a real-time indicator of cleanliness from all biological contaminants and is useful for cleanliness monitoring [4-8]. These data are important because the presence of any organic residue provides a source of nutrients that will later support the growth of even low numbers of microbes present on a surface already cleaned [9]. Although desiccated bacterial cells and spores very rapidly lose their viability, the ATP present in these remains relatively stable [10].

A drawback of using the bioluminescence method is that the surfaces tested are not equally contaminated by human cells, bacterial cells, or other organic material [11]. If low values are measured on one part of the work surface, they cannot be interpreted as applying to the entire surface area [11]. The sampling method is difficult to standardise, and every testing device reads on a specific scale.

Special attention must be paid to defining threshold values, which must be within the effective measuring range of the device [12]. In fact, luminometers are less reliable at the lower end of the measuring range, where the coefficient of variation is the highest. Results outside the effective area are prone to false negatives or else misleading results [13].

The aim of this study was to evaluate the feasibility, usefulness and limitations of a new rapid ATP bio-luminescence method as a real-time surface hygiene monitoring tool in hospital pharmacy cleanrooms. The ATP bioluminescence approach was compared to the traditional microbiological colony count method in order to confirm correlations and to set alert and action limits. To the best of our knowledge, ATP bioluminescence has so far not been used for monitoring hygiene in hospital pharmacy cleanrooms.

Materials and Methods

A total of 2,148 samples taken from four cleanrooms at the Ljubljana University Medical Centre of Pharmacy, Slovenia, between 2016 and 2017 were examined. Three cleanrooms are used for the aseptic preparation of individual therapy: Cleanroom 1 for aseptic preparation (PA), mainly for ophthalmic products, Cleanroom 2 for preparing total parenteral nutrition (PPP) and Cleanroom 3 for preparing cytostatic therapy (PCT). Cleanroom 4 (PI) is used for preparing various parenterals and other sterile solutions with subsequent terminal sterilisation. All rooms are classified as ISO 14644 class 7; EU GMP Grade C, and the laminar airflow chambers are classified as ISO 14664 class 5; EU GMP Grade A.

Room categorisation

We first carried out screening tests by measuring the presence of ATP and colony-forming units (CFUs) on various surfaces in both production and non-production facilities. The rooms were classified into meaningful categories and assigned their corresponding degree of cleanliness. The facilities were divided into production (P) and non-production ones; that is, into cleanrooms (CRs) and not cleanrooms (NE CRs; Table I).

Microbiological analysis

Sampling was performed using the Replicate Organism Direct Area Contact (RODAC) imprint technique. Plates were pressed onto the surface with constant pressure (provided by hand) for at least 10 seconds and transported to the laboratory, where they were kept at room temperature. The plates were incubated for 18 - 24 h at 35 ± 1°C and then for an additional 18 - 24 h at room temperature. In the lab the presence of growth on the media was read daily, and colonies were counted and identified for the species [14].

RODAC plates were prepared and all microbiology samples were evaluated at the Institute of Microbiology and Immunology, University of Ljubljana (IMI), Faculty of Medicine, Slovenia. The microbiological procedures were detailed described previously [14].

To disinfect the used material, a “spray and wipe” technique was used with sterile 70% ethanol (Klerwipe 70/30%, Ecolab, Maribor, Slovenia).

Bioluminescence assay

A Lumitester PD-30 device and LuciPac Pen swabs (Kikkoman Biochemifa Company, Tokyo, Japan) were used. ATP samples were collected with the LuciPac Pen swab by using a plastic mould (6 × 4 cm). Three LuciPac Pen swabs were taken from the surface simultaneously adjacent for each RODAC location, and the ATP measurement was repeated three times. Qualification of the device was done using the Control Kit with a positive and negative control (Kikkoman Biochemifa Company, Tokyo, Japan) and two standards (HyServe GmbH & Co. KG, Uffing, Germany cat. nos. 48-052 and 47-052).

Benchmarking

In line with previous studies [14-17] and considering the room classification, we defined alert and action values for each category for both methods [5]. The defined limits were based on testing the surface samples, which were cleaned by taking into account the best cleaning practices, and they were obtained on the basis of previously performed measurements. The microbiological thresholds were defined according to current standards [18].

Comparison of methods

First, we looked for a correlation between the numerical values. Then the results were evaluated according to the defined thresholds and could be rated as P (Pass)

in the case of compliance or F (Fail) in case of non-compliance. Subsequently, we compared the methods with regard to the performance in finding the inadequate surfaces.

Statistical analysis

The statistical analyses were performed using SPSS software (IBM SPSS statistics, IBM Corp., Chicago, IL, USA).

McNemar's test and Cohen's kappa coefficients were used in the comparison of repeated binary measurements.

Results and Discussion

Rooms were divided into four main categories, according to the room's location, its purpose and the processes taking place there (Table I). Results from ATP and CFU measurements differed greatly due to the random sampling during different parts of the workday and without extensive prior cleaning.

Table I

Room categories with limits for each method: bioluminescent and traditional microbiological RODAC plates

Room categories for method evaluation	Room categories for limit determination	Working place	LuciPac PEN swab values			RODAC plate values
			Pass RLU/20 cm^2	Caution RLU/20 cm^2	Fail RLU/20 cm^2	Fail CFU/20 cm^2
1	1	P/PPP/CR A	35	35 - 70	70	1
	2	P/PA/CR A	35	35 - 70	70	1
	3	P/PI/CR A	35	35 - 70	70	1
2	4	P/PPP/CR	70	70 - 140 (210)	140 (210)	25 (50)
	5	P/PA/CR	70	70 - 140 (210)	140 (210)	25 (50)
	6	P/PI/CR	70	70 - 210	210	25 (50)
3	7	P/PPP/CR F	170	170 - 340 (510)	340 (510)	50
	8	P/PCT/CR F	170	170 - 340 (510)	340 (510)	50
	9	P/PA/CR F	170	170 - 510	510	50
	10	P/PI/CR F	170	170 - 510	510	50
4	11	P/primary packaging preparation/CR	170	170 - 510	510	50
	12	NE CR (working and storage places)	650	650 - 2,000	2,000	50
	13	NE CR (laboratory office)	650	650 - 2,000	2,000	
	14	Other	650	650 - 2,000	2,000	
	15	Hands	2,000	2,000 - 4,000	4,000	

Rooms were divided into four categories: 1 = LAF cabinets in cleanrooms, 2 = cleanrooms, 3 = filters and 4 = other; P = production; CR = cleanroom; CR A = LAF cabinet in cleanroom; CR F = filter (airlock, anteroom); PI = for parenteral preparations; PA = for ophthalmics; PPP = for parenteral nutrition; PCT = for cytostatic therapy.

In our study, in 353 of 537 surface samples (65.7%) no microbial growth was observed. In the first category (LAF cabinets), there were only seven positive samples (6.4%); in the second category (cleanrooms), 20 (13.3%) and in the third category (filters), 19 (27.1%) with microbial growth detected. Only in the last and least stringent category, where all other samples were collected, was microbiological growth found in 138 of 208 samples (66.3%). The results in which microbiological growth was detected and which were nevertheless considered compliant are shown in Table II.

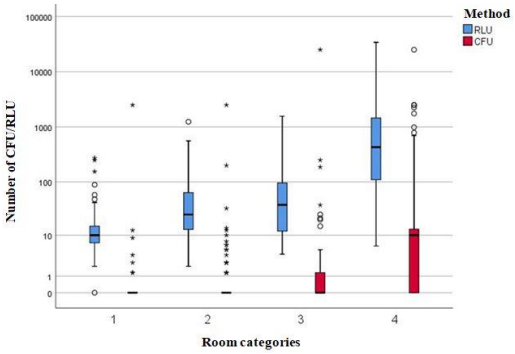
The RLU results are different in terms of negative values. Only 103 of 537 (19.2%) surface sample RLU results have a value less than 10, or equivalent to zero. In the first category there were 56 samples (51.4%), in the second 118 (78.7%) and in the third 55 (78.6%) with positive RLU results. In fourth category, 205 of 208 samples (98.6%) had a measurable RLU result. In other words, only 1.4% of the results were equivalent to 0. The RLU results are shown in Table II. The mean RLU values and CFU values showed, as expected, an increasing trend from the first category (the cleanest one) to the fourth category, where non-sterile production takes place (Figure 1).

Table II

Numbers and percentages of samples that have been assessed as compliant or non-compliant according to RLU or CFU or both (Assessment of the suitability of the result with respect to the set limits)

Room categories for method evaluation	Room categories for limit determination	Working place	Pass RLU Pass CFU		Pass RLU Fail CFU		Fail RLU Pass CFU		Fail RLU Fail CFU		Total
			n	%	*n*	%	*n*	%	*n*	%	*n*
1	1	P/PPP/CR A	46	100.00	0	0.00	0	0.00	0	0.00	46
	2	P/PA/CR A	29	93.55	1	3.23	1	3.23	0	0.00	31
	3	P/PI/CR A	24	75.00	4	12.5	2	6.25	2	6.25	32
Category 1 CR-A (LAF, BVK)		**Total:**	**99**	**90.83**	**5**	**4.59**	**3**	**2.75**	**2**	**1.83**	**109**
2	4	P/PPP/CR	38	92.68	0	0.00	3	7.32	0	0.00	41
	5	P/PA/CR	63	91.30	0	0.00	6	8.70	0	0.00	69
	6	P/PI/CR	34	85.00	1	2.50	2	5.00	3	7.50	40
Category 2 CR (Cleanroom)		**Total:**	**135**	**90.00**	**1**	**0.67**	**11**	**7.33**	**3**	**2.00**	**150**
3	7	P/PPP/CR F	21	91.30	1	4.35	1	4.35	0	0.00	23
	8	P/PCT/CR F	17	94.44	0	0.00	1	5.56	0	0.00	18
	9	P/PA/CR F	22	88.00	2	8.00	1	4.00	0	0.00	25
	10	P/PI/CR F	3	75.00	0	0.00	1	25.00	0	0.00	4
Category 3 CR-F (filter, anteroom)		**Total:**	**63**	**90.00**	**3**	**4.29**	**4**	**5.71**	**0**	**0.00**	**70**
4	11	P/primary packaging preparation/CR	9	100.00	0	0.00	0	0.00	0	0.00	9
	12	NE CR (working and storage places)	67	69.79	3	3.13	24	25.00	2	2.08	96
	13	NE CR (laboratory office)	12	75.00	3	18.75	1	6.25	0	0.00	16
	14	Other	8	13.11	0	0.00	48	78.69	5	8.20	61
	15	Hands	6	23.08	7	26.92	8	30.77	5	19.23	26
Category 4 Other		**Total:**	**102**	**49.04**	**13**	**6.25**	**81**	**38.94**	**12**	**5.77**	**208**
		Total:	**399**		**22**		**99**		**17**		**537**

Rooms were divided into four categories: 1 = LAF cabinets in cleanrooms, 2 = cleanrooms, 3 = filters and 4 = other; P = production; CR = cleanroom; CR A = LAF cabinet in cleanroom; CR F = filter (airlock, anteroom); PI = for parenteral preparations; PA = for ophthalmics; PPP = for parenteral nutrition; PCT = for cytostatic therapy



★ outliers outer fence; ° outliers inner fence

Figure 1.

Results of traditional microbiological (CFU) and bioluminescence methods (RLU) by room category (n = 537)

Rooms were divided into four categories: 1 = LAF cabinets in cleanrooms, 2 = cleanrooms, 3 = filters and 4 = other

Average values, maximum and minimum values, and the standard deviations for each category were calculated. The recommended pass limit values were average values, and the fail limit values were calculated by adding three standard deviation values to the average one. Expressions of "no microbial growth" and "pass limit" do not mean the same value. The intermediate zone between the P and F limits represents the warning zone C (Caution). In the second and third room categories, an additional less stringent threshold was defined (in brackets). The applied limit depended on the height of the sampled location or on the cleaning properties of the sampled surfaces (Table I). This means that for floor contact plates and surfaces that are difficult to clean criteria for a room category with a lower level of cleanliness were used.

Table II and Figure 2 show the results of the assessment and comparison of the methods' ability to predict an inadequate surface.

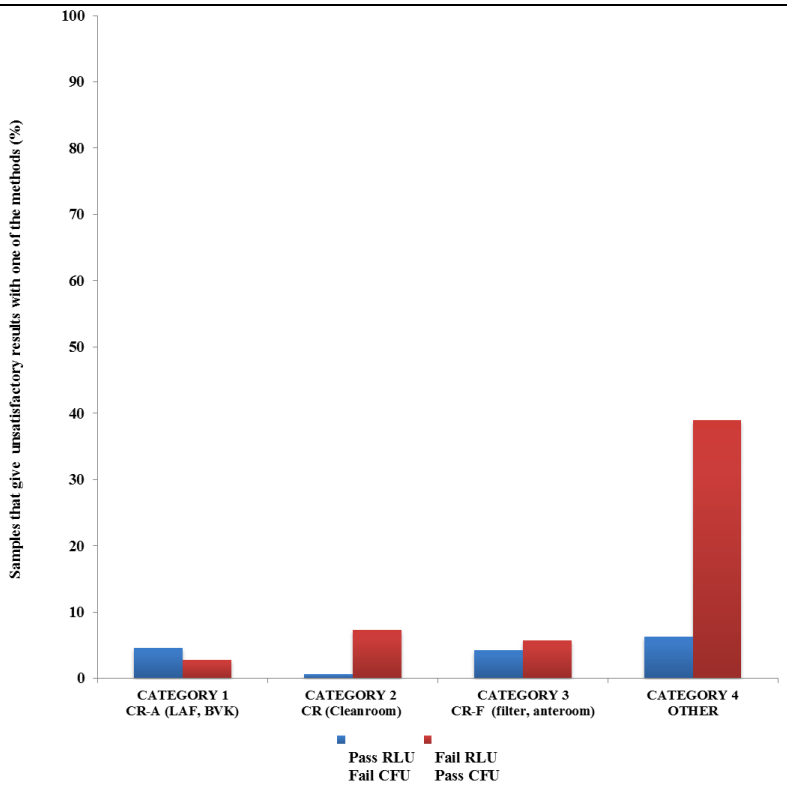


Figure 2.

Percentage of samples that give non-compliant results with one of the methods **regarding** RLU or CFU values

By comparing the predictive value of the method in the first (p = 0.727) and third (p = 1.000) categories, it was statistically impossible to reject the equivalence test, and this confirmed that any differences between the methods were due to coincidence (Table III). In the second (p = 0.003) and fourth (p = 3.6 10^{-13}) categories, we were, however, able to refute equivalence, confirming a statistically significant difference.

Table III

Statistical parameters by individual categories obtained by IBM SPSS statistics

Room category for evaluation methods	*n*	McNemar test p-value	Kappa	Asymptotic standard error [b]	Approximate T [c]	Approximate Significance
Category 1 CR-A	109	0.727	0.296	0.181	3.136	0.002
Category 2 CR	150	0.003 [a]	0.209	0.133	3.448	0.001
Category 3 CR-F	70	1.000 [a]	-0.052	0.02	-0.436	0.663
Category 4 Other	208	3.604E-13	0.017	0.049	-0.035	0.724

Room categories: 1 = LAF cabinets in cleanrooms (CR-A), 2 = cleanrooms (CR), 3 = filters (CRF), 4 = other; [a] = Binominal distribution; [b] = Not assuming the null hypothesis; [c] = Using the asymptotic standard error assuming the null hypothesis

To the best of our knowledge, this study is the first to evaluate the bioluminescence method in hospital pharmacy cleanrooms and define the benchmark for monitoring cleanliness that could indicate the risk from a contaminated environment.

Based on other published comparative studies, we decided to use a device that is capable of measuring the total ATP and providing a strengthened and prolonged signal due to an additional enzyme [10, 12, 19-24].

It was confirmed that values below 10 RLU (LD) are dark noise and negligible. The lowest limit of quantification (LLQ) specified by the manufacturer – that is, 10 fmol (fmol = 10^{-15} mol) of ATP – was also confirmed. The average measured RLU value at this limit was 26.

The approach recommended by Whiteley [11] and Mulvey [15] was adopted to determine alert and action limits. Most of the samples were collected in cleanrooms early in the morning, before the start of routine activities. We assumed that the rooms would be the cleanest at that time and that the effects of detergents and disinfectants would be minimised.

Numerical results were, as expected, practically impossible to compare or correlate because RLU values fluctuated from a minimum of 10 to a maximum of 2,000 RLU *per* sample, and microbial contamination detected by the cultured method was very low and was present in only around 10% of the samples.

However, it should be considered that microbial contamination might have been low due to unsuitable

growth conditions. Although microbes may be alive, they are unable to grow and form colonies [24]. Other reasons for these results might include non-standardised procedures, different methods of handling samples and numerous other factors that can influence the survival and isolation of microorganisms during sampling using traditional methods [25, 26].

To reduce the probability of false negative results, three ATP swabs at each sampling location, for three separate measurements, were taken. By increasing the number of measurements, we reduced the probability of a false negative result [27].

We established the same limits for all the strictest categories (LAF chambers). However, because all results obtained were significantly below the threshold, we decided to lower it further (Table I).

The results obtained by the bioluminescent method largely confirm the data obtained with the classical cultural method. However, despite the agreement of the results obtained, the analysis did not reveal a correlation between them. The results from both methods were quite scattered, with the higher results concentrated in higher room categories, whereas the lower values were in rooms assigned a noticeably lower category, which was expected (Figure 1). A logarithmic comparison of the result values would perhaps have been better; however, owing to a large number of microbiological results with a CFU value of zero, this was not possible.

The main reason for not revealing a direct correlation is to be linked to the approach used: the bioluminescence method determines the total ATP, regardless of its origin. The average ATP load that can be attributed to microbial organisms and was calculated in studies [7, 27] was only 33%. We were aware of this limitation but, due to the stable cleanroom environment, we expected at least a weak correlation, especially in the cleanest categories.

The RLU results may also have been be influenced by some other factors. These include traces of disinfectant (e.g., sodium hypochlorite), eroded surfaces, remnants of plastificators in clothing, ammonia compounds in detergents and so on [27-30]. These factors can modulate enzyme activity, usually reducing it, and can cause cellular lysis by functioning as regents that release ATP. In this study, such influence was minimised by extending the time between cleaning or disinfecting and sampling.

Another important factor is the assumption that all surface contaminants are equally distributed across the entire surface. This factor was minimised by taking an increased number of samples from adjacent locations.

In a number of samples, microbial growth was either completely absent or extremely weak. This may be linked to the sampling itself, culturing or the specific characteristics of the micro-organisms, or to the fact that there is no universal growth medium. It has been estimated that 60% of the microbial biomass on Earth exists in a quiescent state [31]. These micro-organisms are so called VBCN (Viable But Non-Culturable), which is their effective survival strategy. Although, they are metabolically and physiologically active, they do not multiply or grow, even when placed in specific growth media [24]. The microorganisms usually found in cleanrooms are often so weakened from a lack of nutrients, unsuitable conditions and the application of disinfectants that they do not start multiplying even when placed in a growth medium.

By comparing the methods, there were discrepancies associated with predicting inadequate results in room Categories 2 and 4, but in Categories 1 and 3 no differences were found (Tables II and III).

The results showed that significantly more samples were found to be inadequate when assessed using the bioluminescence method. This means that the microbiological criteria leave much room for a negative result, which does not necessarily mean the surface is in fact clean and compliant with the reference criteria. These false negative results imply that many surfaces considered clean and appropriate are not.

Conclusions

This study has confirmed that the RLU method evaluated is suitable and sensitive enough to be used in our cleanrooms. We therefore foresee the bioluminescence method being used as a supplementary method used to assess a room's cleanness before it is used. Perfect information on the quality of the work environment is naturally only possible by using both methods, bioluminescence and traditional microbiology, which still remains the gold standard and provides important qualitative information on the microbiota, providing enough time to successfully implement both curative and preventive measures [14].

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