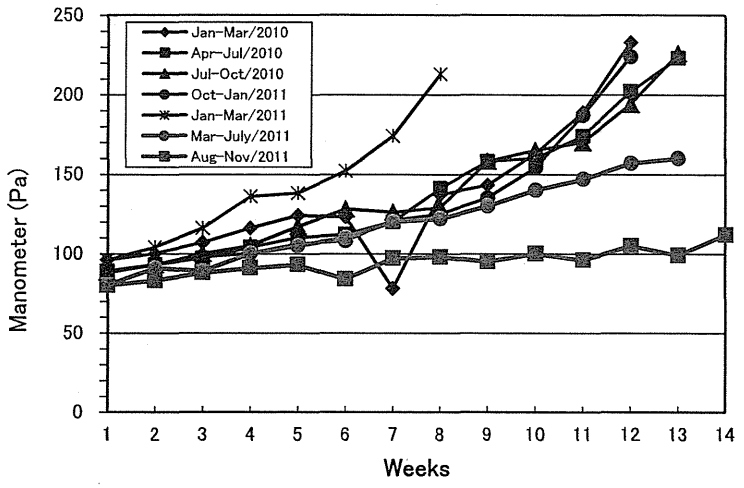

Fig. 3. Manometer Reading of Intermediate filter for P3 lab. Manometer readings before (Black) and after (Red) the installation of water-air filtration system.

Fig. 4. Operation mode display. 1: P3 room pressure, 2: Anteroom pressure, 3: Temperature display

Fig. 5. Inverters I and II

HEPA-filters. Directed flow is further enhanced by the airtight nature of the facility that limits the flow of air to designated routes.

D) Operation features-Special features

a) Eco-run mode- this is a special operation feature that causes the entire facility to go into a power saving mode. It is fully automated and turned on manually when the laboratory is not in use such as night time and weekends. The eco-run mode maintains normal power supply to vital equipment and a minimal supply to those that require power to function but can operate on the minimal power supply when not in active use. It stops the supply of clean air into the ante-room and maintains the temperature in the P3 room at 30°C, hence eliminating the need for air conditioning while saves power consumption in the facility by at least 30%.

b) Computerized Pressure System (Fig. 4)- this is an automatic system that functions in maintaining a negative pressure. It is achieved by maintaining a rate difference between the exhaust and inlet air flow, whereby the exhaust speed is higher than the inlet speed. The efficiency of our system arises from use of two inverter control gadgets inbuilt within the control panel that automatically run inverters at the air inlets, maintaining them at a lower speed relative to exhaust speed (Fig. 5). The gadgets have a display screen and a number of control buttons that allow resetting and calibration of the system without having to modify the

actual inverters. The above features are controlled by a control panel that hosts all automated components in our facility. Operation mode systems are inbuilt within the panel from where they can be operated through a switch on the panel's display. Also built within the panel are air conditioning and negative pressure systems that automatically control the temperature and pressure. The control panel therefore runs the major operation components of our lab while providing a means for continuously monitoring functioning of the facility.

E) Equipment

a) Biosafety equipment

In addition to the usual laboratory equipment, our P3 lab is well equipped with sufficient biosafety equipment. There are three biosafety cabinets (BSCs); two of Class II AB and one of Class II B2. The Class II B2 is an exhaust type that allows manipulation of highly infectious agents. Class II AB biosafety cabinets offer primary containment and are used when manipulating less hazardous agents. Two autoclaves are provided to aid in waste disposal and infection prevention. They have different operating programs to allow for the decontamination of a range of wastes with varying levels and the nature of contaminants. Finally, the lab is sufficiently supplied with all the necessary Personal Protective Equipment (PPE). People using the lab are required to put on a full set of PPE upon entering the anteroom and are required to remain dressed until exit the facility (Fig. 6).

b) Emergency response equipment

These are gadgets that are meant to enable users and the rest of laboratory workers to respond to accidents mainly fires, power failures and gas leakage. The facility has two fire extinguishers; a carbon dioxide type in prep room 2 and a powder type in the P3 lab. These are regularly inspected and refilled, strategically stationed and have a user instruction manual attached. A gas detector and fire alarm are also provided. The gas detector is fixed on top of BSC Class II B2 and has an alert system that goes off in case of any gas leakage. The fire alarm is inbuilt within the lab from where it activates fire alerts at the onset of a fire, signaling the laboratory staff to respond appropriately. Our institution is not ignorant of the possibility of a complete power failure, by both generators and commercial power lines. A fluorescent lamp supported by a rechargeable battery is therefore built within our lab to provide emergency lighting. The bulb automatically switches on for 1h, following a total power failure to allow users to finish-up their experiments or undo set-ups and evacuate.

Fig. 6. P3 Lab user in full PPEs. 1: Gloves (double), 2: Face Shield, 3: N95 mask, 4: Wrist band, 5: Front covered gown, 6: Coverall, 7: Safety boots

MAINTENANCE

Maintenance is a key aspect of biosafety management systems because use of faulty and unconditioned machines can cause contamination. The P3 laboratory is quite sensitive due to the nature of the biohazards it handles hence regular maintenance is of the utmost importance. Maintenance schedules ranges from daily to yearly. Regular maintenance involves;

a) Daily- This includes floor cleaning, door and door handle disinfection, recording of various vital parameters, documentation of observed off-readings and waste management. Additionally, members of the staff are required to turn on the BSC UV light to decontaminate the cabinet. The P3 room is also decontaminated daily after work for at least an hour using preset UV lights.

b) Weekly- mainly involves the cleaning and changing of pre-filters (Fig. 7). Being part of the initial air filtration stages, the fibrous pre-filters trap the bulk of fine air contaminants (Fig. 8). It is therefore necessary to change them every week to eliminate chances contaminating or damaging the entire system. The generator is also maintained at weekly intervals. It is supported by a control panel that is automatically set to turn on the generator on Monday morning between 9:30 am and 9:40 am to allow for routine maintenance, checking and recording of vital operating parame-

Fig. 7. Lab staff changing pre-filters

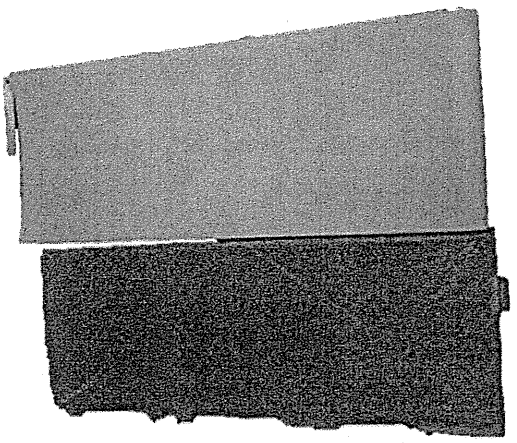

Fig. 8. Clean and soiled pre-filters

ters and battery recharge.

c) Monthly- maintenance is routinely performed mainly on the air filtration system depending on manometer readings. It involves replacement of intermediate filters which get soiled in the course of its usage. The manometer monitors the condition of these filters and indicates when they are due for replacement (Fig. 9). The black arm of the manometer shows meter readings, indicating how much of the intermediate filter has been consumed while the red arm shows maximum consumption, at which time the intermediate filter should be changed (Fig. 10, 11).

d) Yearly- servicing is performed by experts from Japan during which the overall working condition of the facility is assessed and necessary corrective measures undertaken. BSCs are fumigated and HEPA filters are changed alongside minor repairs and improvement of other components of the facility. BSCs are fumigated using paraformaldehyde powder and 100 ml of water for 1 h in a beaker and neutralized using ammonium hydrogen carbonate (Fig. 12). Train-

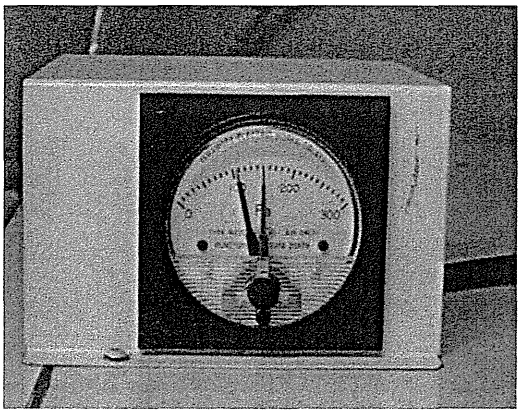

Fig. 9. Manometer of intermediate filter

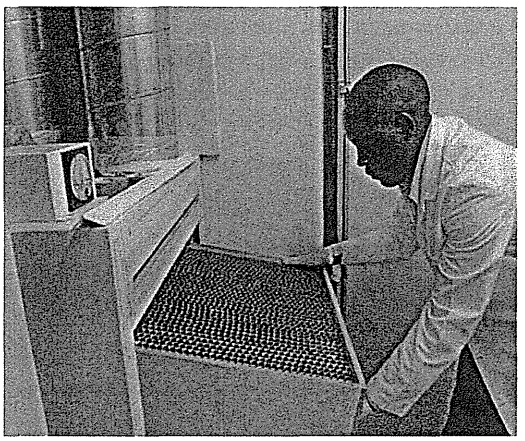

Fig. 10. Lab staff removing used-up intermediate filters

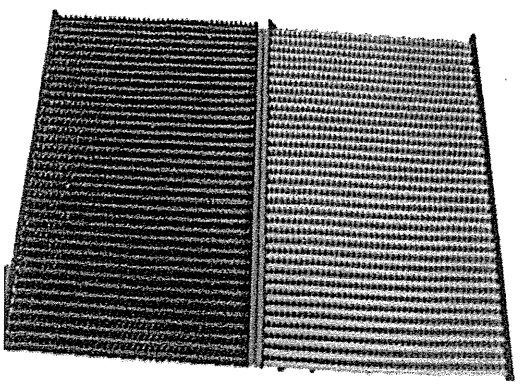

Fig. 11. Used-up and clean intermediate filters

Fig. 12. Positioning of hotplates and sealing biosafety cabinets in preparation for fumigation

ing of NUITM laboratory staff in some core maintenance procedures is ongoing to permit our facility to perform more regularly with minimal maintenance costs (Fig. 13-A, 13-B, 13-C, 14-A, 14-B).

SECURITY FEATURES

Operations in the P3 lab call for adherence to strict security measures. In the first place, the lab is located in the rear end of the CMR lab where movement by other laboratory workers is minimal. Further, only authorized persons can access the laboratory, a measure that is further enforced by using secret opening passwords that are regularly changed (Fig. 15). New facility users must be trained or accompanied by our trained staff. There is a laboratory registration record that summarizes their activities in the laboratory, which can also be used for follow-up in case of any irregularity.

DOCUMENTATION

Documentation is a critical feature for the maintenance of safety standards. Our institution has variety of records for users and activities within the P3 facility. Generally, these records track activities in the P3 lab, movement in and out of the laboratory and the usage of laboratory equipment.

a) P3 lab in/out record- This is a record of persons who are working in the P3 facility. It captures daily activities in the P3 lab, the number of people using it and the usage of BSC.

b) BSC usage record- This record is used to monitor the usage of BSC which are core components of a P3 lab. It captures information on person, time and pathogens being handled in the facility. Apart from tracking usage of BSC, it can also be used to draw up a roster for smooth sharing of the facility among a number of research teams. The record can also be used to schedule maintenance activities.

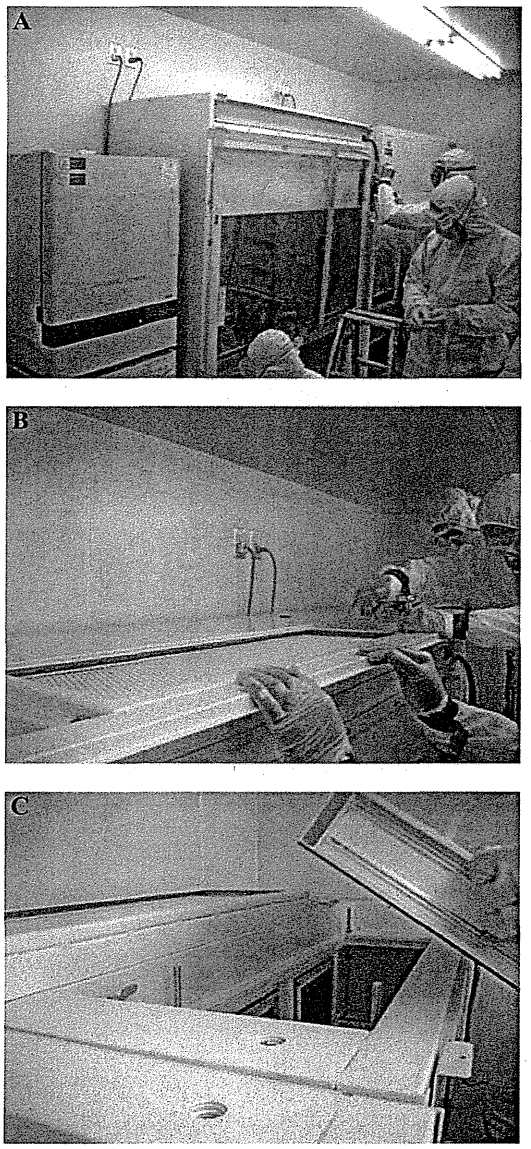

Fig. 13. A: Sealing of BSC for Formalin Fumigation, B: Removing of old HEPA filter from BSC, C: Replacing of new HEPA filter into BSC

c) Daily check-points in ante-room- In the ante-room the daily check-points on observing the monitoring of the overall working conditions of the P3 lab. The parameters are shown on the control panel such as room temperature, negative pressure etc.

d) Standard operating procedures (SOPs)- Failure to adhere to good laboratory practices, laboratory worker errors and the misuse of equipment accounts for the majority of laboratory injuries and laboratory-acquired infections. Consequently, SOPs and standard laboratory practices must be

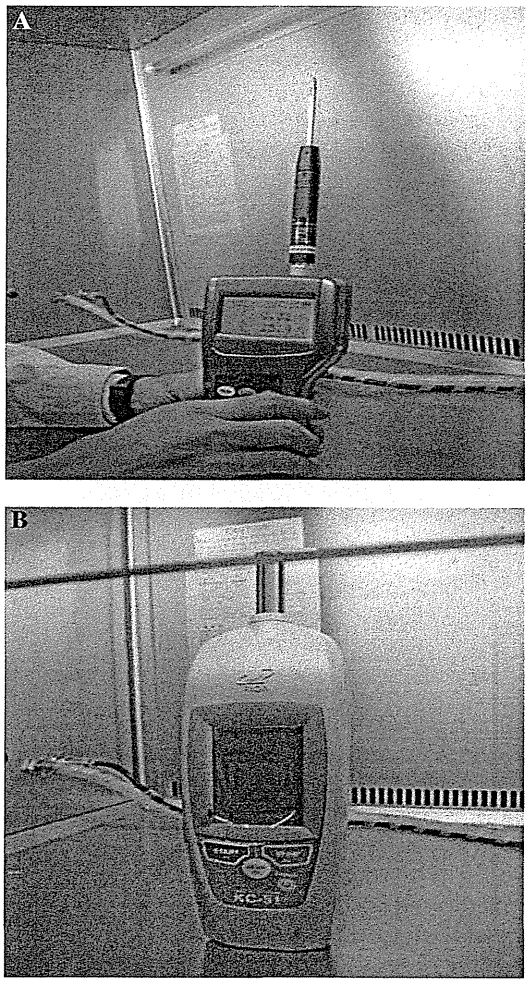

Fig. 14. A: Velocity testing in BSC, B: Particle counting in BSC

Fig. 15. Multiple signage. 1: Activated pathogens symbol, 2: Biohazard sign, 3: Lock pad (a security feature)

in place to prevent laboratory-acquired infections, minimize laboratory accidents and elevate biosafety and security measures. Our facility has adopted and enforced a biosafety and operation manual with reference to the WHO manual for safety applications which is used to inform and guide all operations within the P3 facility. Our facility maintains an SOP for each equipment within the P3 lab, for use within the lab and also for training purposes. SOPs provide a brief description of the equipment, how to operate it and calibration routines where applicable. Apart from internal SOPs, researchers from other institutions are required to develop and submit their project's SOPs well before they are permitted to commence any research activity in order to facilitate risk assessment procedures.

BIOSAFETY SYMBOLS

Our facility has a number of signage features that are located strategically in adherence to biosafety standards and specifications. First, there is an emergency exit indicator signaling a safe exit route through the back door in the case of an accident (Fig. 16). Biohazard signs, which are also located in relevant areas, indicate the need for extra caution. There is also an activated pathogen signage feature that alerts users to the possible presence of active pathogens at any given time (Fig. 15). This helps in preventing would-be infection and contamination of research materials. Further, there are access signage for areas of limited entry and those with special access conditions. For example, the P3 area has a signage that limits unnecessary traffic while the P3 lab main entrance has a signage indicating limited entry, indi-

Fig. 16. Fire exit sign

cating that only authorized persons are permitted to enter the laboratory. The facility's signage is openly displayed on all relevant areas. Most importantly, areas of multiple hazards are clearly indicated using multiple signs. All signage features follow universal specifications in terms of color scheme and images.

WASTE MANAGEMENT

Because the laboratory generates a variety of infectious wastes, the need exist for a proper waste disposal system in order to minimize the potential for exposure of laboratory workers who must handle these materials. Our institution therefore follows waste disposure procedures as provided in the KEMRI waste disposal guidelines and procedures protocol. The protocol classifies wastes into various categories, provides a unique color of waste containers and packaging and provides a set of handling and disposal policies for each category. A set of Policies are provided for each category, instructing users on how to handle and dispose of various types of wastes. Appropriate labeling is emphasized as a means of communicating to staff on the type of waste being handled while informing waste disposal activities. Our P3 lab is well equipped with all the necessary disposal equipment. Persons generating infectious wastes are responsible for preparing the wastes for eventual disposal through standard procedures. Following initial treatment, wastes materials are disposed of as per protocol by designated staff.

TRAINING

Usefulness, efficiency and safety at our facility depends on the level of awareness and expertise among researchers using P3 facility, hence the need for training. The training component in our P3 lab is of utmost importance not only because of the capacity development but also because of the relevance of P3 facilities in transforming research, since this is a relatively new concept in Kenya (Fig. 17-A, 17-B). Since 2007, the facility has trained about 70 researchers, equipping them with necessary knowledge and skills to independently work in a P3 lab. The effectiveness of the training sessions is evaluated through a standard examination in which the outcome informs the institution on existing gaps of knowledge and areas of weakness. The institution is therefore able to continuously improve the curriculum while improving training outcomes. P3 training is offered in the form of a continuing education course for fresh scientists who might need to use the facility in the future, NUITM staff and other users. Training is carried out through an annual two-day workshop with the help of a

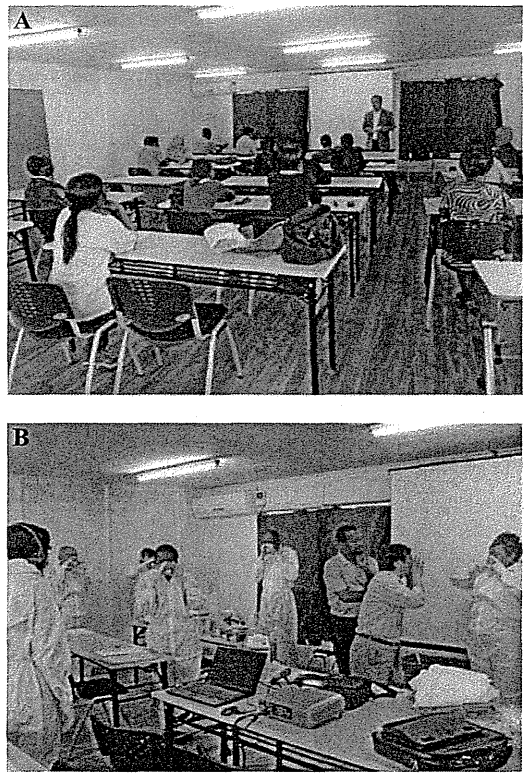

Fig. 17. A: P3 Lab Training seminar (Lecture), B: P3 Lab Training seminar (Practicals)

short curriculum presented in the form of lectures, demonstrations and practicals. Participants are first introduced to the concept of biosafety and P3 facilities before being taught about the components of the laboratory and how to use them. Most specifically, training focuses on informing participants on the hazards associated with the facility and possible ways of minimizing personal harm while minimizing the risk of exposing other people to danger. Our staff also conducts refresher training for post-trained researchers before using the facility. This is basically a remedial on laboratory rules and regulations, good practices and safety procedures.

BIOSAFTY MEETINGS

These are meetings, which are held on the last Friday of every month brings together all researchers who have used the P3 lab within that particular month and other trained users (Fig. 18). The objective of meetings are to provide a forum for attendees to share their experiences with regards to using the P3 lab, identify and discuss areas of difficulty, update each other on upgrading or new installations and to provide information concerning ongoing activities.

Occurrences of alarms or system breakdowns are informed and discussed to identify their causes and generate preventive procedures for these and other similar occurrences. Attendees are also reminded of basic safety rules and practices, emphasizing on the need to observe personal safety and safety of other people around the institution. It is mandatory for all users and trained staff to attend and participate in the meeting.

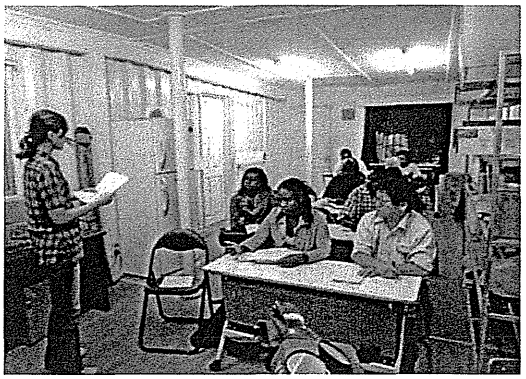

Fig. 18. Monthly Biosafety Meeting

USAGE OF THE P3 LABORATORY

The P3 lab has been in use since 2007 although active use began in 2010. A wide variety of studies have been carried out in the lab and some are still going on. Users of the laboratory are KEMRI-NUITM staff and researchers from other centers of KEMRI. Research activities carried out in the lab can be large scale studies or short-term projects by individual researchers or students. Our staff also uses the

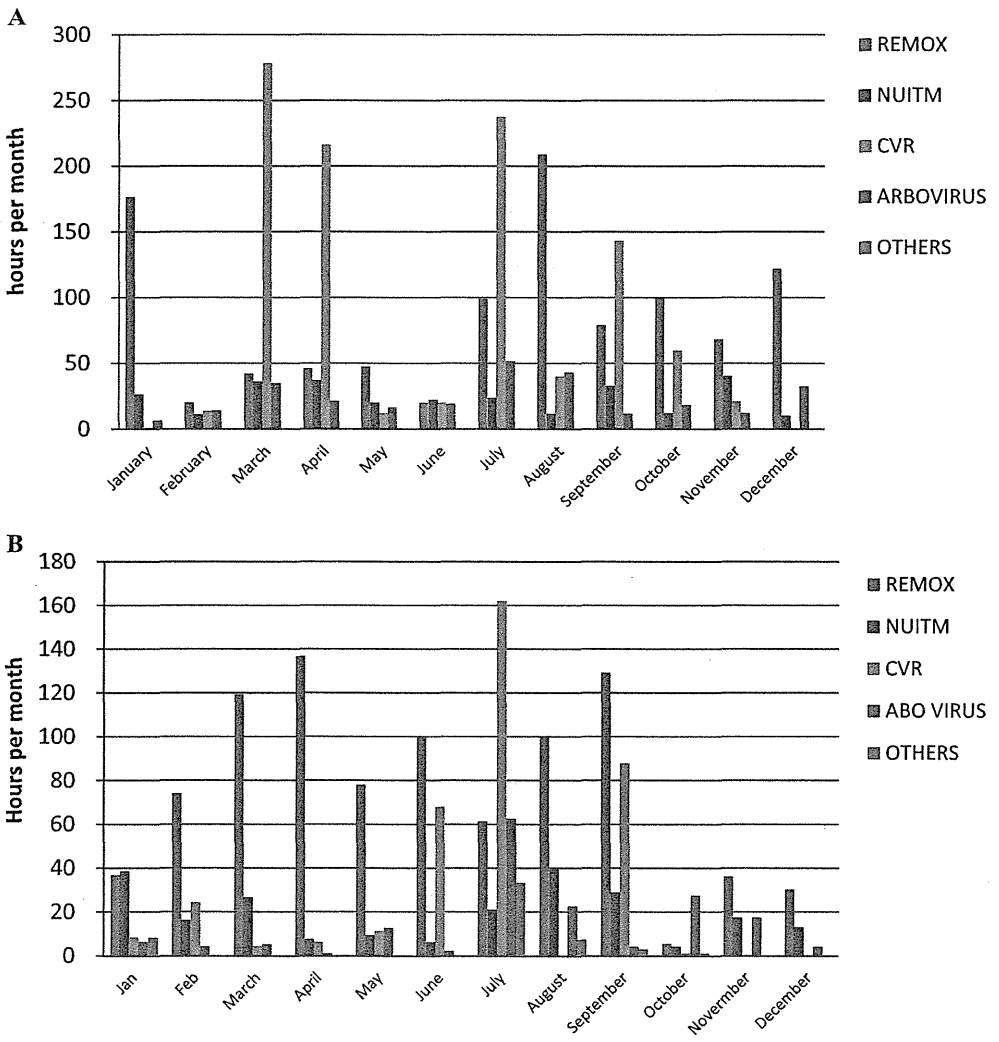

Fig. 19. A: Monthly working hours in P3 Lab in 2010. B: Monthly working hours in P3 Lab in 2011.

laboratory to process BSL-3 biological materials that are encountered in the course of routine research activities. To date, the majority of users of our facility have been research teams from other institutions, as shown in the graphs below, with the Remox project (CRDR-KEMRI) and CVR-KEMRI teams being the heaviest users (Fig. 19-A, 19-B). Through the entire course of its usage, a large number of pathogens have been isolated and/or propagated in the P3 lab. These include *Salmonella typhi, Bacillus anthracis, Vibrio cholerae,* yellow fever viruses and some strains of MDR-TB, among others. Bio-containment facilities in our laboratory have been of great use in enabling research activities involving such hazardous agents.

ACCREDITATION

Our facility is firmly established as per standard safety and security specifications [4] and has been in use for quite some time now. Operations within the P3 lab are in accordance with biosafety procedures, complete with standard operating procedures and necessary documentation as a prerequisite of accreditation of the facility. Currently, our institution is fully certified through the manufactures and is in the process of acquiring accreditation from local and international bodies.

CONCLUSION

In conclusion, our P3 facility is a comprehensive research unit that has continued to project our institution into a center of excellence in various fields of research. Most significantly, it has fully solved the problem of laboratory-acquired infections and threats posed by the manipulation of hazardous biological materials in an unsecured system. The facility will continue to positively influence research experiences and outcomes while developing research capacity for the prevention and management of tropical diseases.

ACKNOWLEDGMENT

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Alarin but not its alternative-splicing form, GALP (Galanin-like peptide) has antimicrobial activity

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ABSTRACT

Alarin is an alternative-splicing form of GALP (galanin-like peptide). It shares only 5 conserved amino acids at the N-terminal region with GALP which is involved in a diverse range of normal brain functions. This study seeks to investigate whether alarin has additional functions due to its differences from GALP. Here, we have shown using a radial diffusion assay that alarin but not GALP inhibited the growth of *Escherichia coli* (strain ML-35). The conserved N-terminal region, however, remained essential for the antimicrobial activity of alarin as truncated peptides showed reduced killing effect. Moreover, alarin inhibited the growth of *E. coli* in a similar potency as human cathelicidin LL-37, a well-studied antimicrobial peptide. Electron microscopy further showed that alarin induced bacterial membrane blebbing but unlike LL-37, it did not cause hemolysis of erythrocytes. In addition, alarin is only active against the gram-negative bacteria, *E. coli* but not the gram-positive bacteria, *Staphylococcus aureus*. Thus, these data suggest that alarin has potentials as an antimicrobial and should be considered for the development in human therapeutics.

1. Introduction

In recent years, research in the field of novel antimicrobial peptides has intensified due to the need for newer effective antibiotics to overcome resistance issues of conventional antibiotics. Antimicrobial peptides, isolated from various bacteria, fungi, plants, invertebrates and vertebrates are important components of natural defenses of most living organisms [1]. These molecules have the added advantages of being very small in size, amphipathic and positively charged which allow them to bind and disrupt microbial membranes [2]. Some effective antimicrobial peptides reported thus far include human LL37 which is active against *Staphylococcus aureus* [3,4] and *Escherichia coli* [5] and β-defensins against *Enterococcus faecalis* and *Helicobacter pylori* [6–9].

The galarin family of neuropeptides consists of galanin, a galanin-like peptide (GALP) and a newer member called alarin. Human galanin consists of 30 amino acids and is encoded by the *GAL* gene. GALP consists of 60 amino acids and it can activate galanin receptors (GalRs) because residues 9–21 of GALP are identical to the first 13 amino acids of galanin [10]. Alarin consists of 25 amino acids and is derived from an alternative-splicing of the *GALP* gene that excludes exon 3. Its precursor consists of the signal sequence of the prepro-GALP, the first 5 amino acids of the mature GALP peptide and another 20 amino acids that are not identical to any other peptides. Unlike galanin and GALP, alarin does not bind to GalRs [11,12].

Galanin and GALP mRNAs are widely distributed in CNS as well as in the periphery in GIT, heart, dermis, epidermis, nerves, bone and joint tissues [13]. Alarin mRNA was first detected in ganglionic cells of neuroblastonic tumors [11] and it has a much wider CNS distribution than GALP [14]. It can also be found localized around blood vessels in the skin [11]. Galanin and GALP are involved in a diverse range of normal brain functions such as feeding and

Abbreviations: GALP, galanin-like peptide; *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*.

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metabolism, osmotic regulation and water intake, nociception, neuronal injury, survival, regeneration, and neuroprotection, learning and memory and anxiety-related behaviors [13]. Like galanin and GALP, alarin is involved in feeding behavior [12,15,16], food intake and reproductive hormone secretion [12,16,17]. It also has vasoactive and anti-inflammatory activities [15]. The relationship between alarin and GALP is evolutionally and functionally interesting. Alarin shares only 5 conserved amino acids (APAHR) of the N-terminal region with GALP, while all other residues of the C-terminal region are very different. Hence, it is interesting to uncover other novel and specific functions of alarin arising from the differences in amino acid sequence from GALP. This paper investigates the antimicrobial activity of alarin and GALP against *E. coli* and *S. aureus*.

2. Materials and methods

2.1. Materials

Human LL-37, human GALP (galanin-like peptide), and human alarin were purchased from the Peptide Institute, Inc (Japan). Truncated peptides of human alarin [alarin(6–25), alarin(11–25) and alarin(16–25)] were synthesized and purified by IBL co. (Japan). Trypto-Soya Broth was purchased from Nissui (Japan).

2.2. Radial diffusion assay

The antibacterial activities of human LL-37, human GALP (galanin-like peptide), human alarin, and its truncated peptides were all evaluated by radial diffusion assay [18,19], a modification of the sensitive assay for antimicrobial peptides described by Lehrer and colleagues [20,21]. Briefly, to obtain bacteria growth in the mid-logarithmic-phase, an overnight bacterial culture was diluted 1:1000 in Tryptic soy broth (TSB) and was incubated at 37 °C until the optical density of the aliquot reached an absorbance value of 0.4 measured at 620 nm wavelength. The bacteria suspension was centrifuged at 900*g* for 10 min at 4 °C, washed once with ice-cold 10 mM sodium phosphate buffer (SPB; pH 7.4), and was re-suspended in ice-cold SPB. Based on previously prepared standards of the optical density at 620 nm wavelength, a volume containing 1×10^6 bacterial CFU was added to 10 ml of previously autoclaved 10 mM SPB containing 3.0 mg of TSB medium, 1% low-electroendosmosis-type agarose (Sigma), and 0.02% Tween 20.

After rapid dispersion of the bacteria, the agar was poured into an agar plate to form a uniform layer of approximately 2 mm deep and was punched with a 3-mm-diameter gel punch to make evenly spaced wells. Following the addition of 5 μl of antimicrobial agents to each well, the plates were incubated for 3 h at 37 °C and were then overlaid with 10 ml of sterile agar consisting of 6% TSB (double-strength solution) and 1% agarose. Antibacterial activity is identified as a clear zone around the well following incubation for 18–24 h at 37 °C and is measured as the difference in the diameters of the clear zone around the wells containing the antimicrobial peptides and buffer control (3 mm). These experiments were repeated four times.

2.3. Electron microscopy

The effect of LL-37 and alarin on the morphology of *E. coli* was evaluated by scanning electron microscopy. Briefly, the bacteria were treated for 2 h with 20 μM LL-37 or alarin on a MAS coated slide glass (Matsunami co., Japan), air-dried and then fixed with 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4). Specimens were further fixed in 1% osmium tetroxide and dehydrated through a graded ethanol series (50%, 70%, 80%, 95%, and 100%) and isopentyl acetate. The dehydrated specimens were then mounted on steel stubs, sputter-coated with a mixture of gold/palladium (Joel JFC-1100), and imaged using a Jeol JSM-840A electron microscope (Jeol, Japan) at 8 kV.

2.4. Hemolysis

Horse blood was purchased from Nihon Bio-Test co. (Japan). Horse blood erythrocytes were rinsed three times in phosphate buffer saline (PBS) by centrifugation at 1000*g* for 10 min and then re-suspended in 8% (vol/vol) PBS. Next, a 50 μl erythrocyte suspension was mixed with 50 μl LL-37, alarin, or GALP and incubated for 1 h at 37 °C. Tween 20 at 2% served as a positive control. The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm wavelength and this is expressed as a percentage of the value of Tween 20-induced hemolysis.

2.5. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), with Dunnett's Multiple Comparison Test to identify between-group differences using the GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA), with p values < 0.05 considered as significant. Statistical significance is expressed as $^*p < 0.05$ from data ($n = 4$).

3. Results

The antimicrobial activity of alarin (200 pmol) was compared to its alternative splicing form, GALP and a well-studied antimicrobial peptide human cathelicidin, LL-37. Alarin inhibited the growth of *E. coli* (ML-35), with an increase of ~3.5 mm in the clear zone diameter in comparison to the buffer control (Fig. 1A and B). This inhibitory activity of alarin was also comparable to LL-37 at 200 pmol where its clear zone diameter had an increase of 4 mm when compared to the PBS buffer control (Fig. 1A and B). Its alternative-splicing form, GALP, however, did not show any antimicrobial activity against *E. coli* where the clear zone diameter remained the same as that of the buffer control (Fig. 1A and B). To examine further the dose–response of the antimicrobial activity of alarin; 25, 50, 100 and 200 pmol alarin were used for a radial diffusion assay. Alarin showed significant dose-dependent increases in antimicrobial activity against the gram-negative bacteria, *E. coli* (Fig. 1C). However, a dose up to 200 pmol alarin did not show any antimicrobial activity against the gram-positive bacteria, *S. aureus* (Fig. 1D). LL-37 in micromolar concentrations have been reported to induce bacterial membrane blebbing, leading to a leaky membrane and cell death in *Burkholderia pseudomallei* and *Burkholderia thailandensis* [22,23]. Using electron microscopy, we showed that 20 μM LL-37 induced extensive membrane blebbing on *E. coli* (Fig. 2B) compared to the buffer control (Fig. 2A). Membrane blebbing was also observed on *E. coli* incubated with alarin at 20 μM but is less extensive than those caused by LL-37 (Fig. 2C).

To explore the role of the N-terminal region of alarin, antimicrobial activity of truncated peptides of alarin, alarin (6–25), alarin (11–25), and alarin (16–25) were examined by a radial diffusion assay (Fig. 3). Alarin (6–25) and alarin (11–25) showed weak antimicrobial activity, while the antimicrobial activity of alarin (16–25) was completely abolished (Fig. 3). These results suggest that both the C-terminal and N-terminal regions of alarin are essential for its strongest killing effect.

It was reported that LL-37 has hemolytic activity on erythrocytes [22]. To determine the hemolytic activity of alarin and GALP, 10 μM of each peptide were incubated with horse erythrocytes for