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Determination of the Infective Dose of *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853) When Injected Intraperitoneally in Sprague Dawley Rats

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Authors' contributions

All authors contributed significantly to the study. Author SJ developed the planning protocols and executed the studies under the mentorship of authors LPP, ASA and GP. Author JJ, a veterinarian, monitored the animals. Authors JJ and RS performed the histological examination of tissues and managed animal post mortems. Author PA supervised the microbiological aspects while author ASA handled the technical and editorial aspects of the study. All authors read and approved the manuscript.

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ABSTRACT

Objective: To determine the infective dose of *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853) in Sprague Dawley rats, when the inoculum is injected via the intraperitoneal route.

Design and Methods: In interest of animal welfare and to reduce the number of animals used, we utilized the "up-down procedure" for dose determination of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Animals were grouped in fours, injected IP with inoculums of known concentration of a single organism and observed for 10 days. Clinical signs such as lethargy, increased respiration, porphyrin staining was recorded. Gross necropsy was performed ten (10) days post infection. Only one infective dose was done at a time and depending on the outcome, the size of inoculum was adjusted for the next step in the experimental infectious process.

Results: Doses of inoculum was carefully titrated and the highest tolerable dose for each organism was determined. These doses allowed for survival of the animals and gave clinical signs, which mimicked the scenario in a human population. Symptoms of infection included lethargy, ruffled fur, porphyrin staining, dehydration and hunched back. At necropsy at 10 days post infection, common indicators of infection observed were ascites, abscesses on the intestinal wall, kidneys, liver and spleen. There were also fibrin tags, rounded livers, enlarged spleen and increased pericardial effusion was prominent in the *S. aureus* infected group.

Conclusions: The infective doses were determined based on clinical signs, survival and post mortem changes. The doses determined for *Staphylococcus aureus* (ATCC 29213) was 1.75 X 10^{10} cfu/ml and *Pseudomonas aeruginosa* (ATCC 27853) was 3.0 X 10^{8} cfu/ml. These doses would be useful in infective animal studies to determine the efficacy of antimicrobial agents.

Keywords: Infective dose; Pseudomonas aeruginosa; Staphylococcus aureus; clinical signs.

1. INTRODUCTION

Pathological conditions must be studied and understood before viable methods of treatment can be developed. It is therefore necessary to create models of animal infection which can closely mimic the condition as it would occur in humans. This is if great importance especially when it is not practical or unethical to use humans as test subjects. Animals are biologically similar to humans and as such, are susceptible to many of the same disease conditions which afflict humans. In addition, their environment can be easily controlled (diet, temperature, lighting) which would be difficult with humans. The most important reason however is that it would be unethical to deliberately expose humans to health risks in order to observe the course of a disease.

Since the work of pioneers such as *Jenner and Pasteur* [1] more than a century ago, animal models have provided essential information in the study of infectious diseases. Although much experimentation can now be accomplished with *In-vitro* systems, animals continue to be necessary for studying the processes of inflammation, specific infectious diseases, and pharmacologic treatment of infectious diseases. The use of whole animal models is considered important to the understanding of the very complex temporal relationships that occur in infectious disease involving the body, its neuroendocrine and immune systems, as well as the infectious organism.

It is therefore imperative that the disease condition be replicated in an animal model before any agent is tested. In the case of infective disease studies, the dose of the infectious agent is of paramount importance. The dose administered must produce the desired diseased state before a new treatment option can be tested. From an animal welfare point of view, it is important to use as few animals as possible and the pain endured by the animal be kept at a minimum. For all infectious disease research including virulence tests in animal models, endpoints should be established that minimize the potential for pain and/or distress in the animals [2]. There are various methods described for determining the lethal dose and LD_{50} of an infective agent such as Reed-Muench [3], Litchfield and Wilcoxon [4], Miller-Tainter [5], Lorke [6] and Karber [7] methods. Classic LD_{50} methods were developed in the 1920s and required the use of 100 animals for 5 dosage groups. This was reduced by the *Organization for Economic Cooperation and Development* (OECD) in 1981 to 30 animals, and further

reduced to 20 animals in 1987 [8]. *Fund for the Replacement of Animals in Medical Experiment* (FRAME) believes that the lethal dose test is unnecessarily cruel and scientifically invalid. The test involves giving groups of animal doses of a test substance until it kills half of the study population. Several countries including the United Kingdom, have taken steps to ban oral LD_{50} and the OECD, the International governments advisory body abolished the requirement for the oral test in 2001 [9]. Although we chose the intraperitoneal route as our site of infection as opposed to the oral route, we still complied with the guidelines for good animal handling and within international guidelines for welfare of animals used in research.

2. METHODOLOGY

2.1 Materials

Female Sprague Dawley rats (200-250 g) were obtained from the Animal House, School of Veterinary Medicine (SVM), University of the West Indies (UWI), Trinidad. Animals were weighed, tagged and individually caged in an allocated animal research room at the SVM. Animals were allowed seven (7) days to acclimatize to the local conditions of the room before use in the study. During the acclimatization period, animals were maintained daily by the same personnel.

2.2 Housing and Feeding Conditions

The temperature in the experimental animal room was ambient. Lighting was set to the sequence of 12 hours light, 12 hours dark. For feeding, conventional laboratory diets were used with an unlimited supply of food and water for drinking. Animals were individually housed in metabolic cages.

2.3 Preparation of Animals

The animals were, randomly selected, weighed and tagged to permit individual identification. Animals were weighed daily during the acclimatization period, and after this initial seven (7) day period, they were infected with a given dose of the pathogen. Animals were returned to their allotted cages and observed for 10 days post infection. All animals were weighed daily and all mortalities, clinical signs, time of onset and duration of illness were recorded. Gross necropsies were performed on all animals found moribund, and on all survivors at 10 days post infection.

2.4 Up Down Procedure (UDP) OECD

In the up down procedure, animals are dosed one at a time. If the animals survived and there were no clinical signs of infection, the dose for the next group was increased. Each animal was observed for 48 hours, and then for 10 days for delayed death. If the animals died, then the dose for the next group was decreased. This method allowed for "titration" of the optimal dose which yield the clinical condition required. The clinical signs monitored during the infective studies include abdominal breathing, abdominal wasting, diarrhea, increased respiration, lethargy, loss of righting reflex, lying on sides, porphyrin staining, tremors and vent staining.

2.5 Test Organisms

The test strains of *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) were obtained from the Microbiology Department of the Eric Williams Medical Sciences Complex. Organisms were cultured onto blood agar plates to ensure purity of growth and stored in Brain Heart Infusion (BHI) broth with glycerin at -80℃. As a quality control measure, the organisms were subsequently identified and susceptibility pattern determined by using Dade Behring Microscan.

2.6 Inoculum Preparation

BHI containing the test organism was allowed to thaw to room temperature, cultured onto blood agar plates and incubated for 18-24 hours at 37℃.Inoculum was prepared by suspending individual colonies in normal saline 0.9%. The aim was to produce a standard inoculum of 1.0 X 10^{10} cfu/ml for *Staphylococcus aureus*, and 1.0 X 10^{9} cfu/ml for *Pseudomonas aeruginosa*. The dilution factor which was required to reduce these concentrations to a 0.5 McFarland standard (MFS) (1.5 X 10^{8} cfu/ml) was calculated as shown in Tables 1 and 2. Depending on the dose required, the volume of the prepared inoculum was determined.

A nephlometer was used to confirm the concentration of the organism after the dilution step as being equivalent to the 0.5 MFS. As a quality control measure, serial dilution was performed on the test dose and ten (10) microliters was plated onto a blood agar plate. This was then incubated for 18-24 hours at 37℃. Colony counts were performed to ensure the inoculum contained the desired concentration of the organism.

Table 1. Preparation of inoculum for infection with *Staphylococcus aureus* (ATCC 29213)

Concentration CFU/ml	Volume/ml of $1.0X10^{10}$ cfu/ml used	Dilution factorfrom $1.0X10^{10}$ cfu/ml to 0.5 MFS	Volume of suspension/µl	Volume of NaCl 0.9%
1.0 X 10^{10}	1.0	100/1.5= 66.7	100	6.57
1.5 X 10^{10}	1.5	100/1.5= 66.7	100	6.57
1.75 X 10^{10}	1.75	100/1.5= 66.7	100	6.57
1.87 X 10^{10}	1.87	100/1.5= 66.7	100	6.57
2.0 X 10^{10}	2.0	100/1.5= 66.7	100	6.57

Table 2. Preparation of inoculum for infection with *Pseudomonas aeruginosa* (ATCC 27853)

Concentration CFU/ml	Volume/ml of 1.0 X 10^9cfu/ml used	Dilution factor from $1.0X10^8$ cfu/ml to 0.5 MFS	Volume of suspension/ml	Volume of NaCl 0.9%
1.0 X 10^8	0.1	10/1.5= 6.67	1	5.57
2.0 X 10^8	0.2	10/1.5= 6.67	1	5.57
3.0 X 10^8	0.3	10/1.5= 6.67	1	5.57
5.0 X 10^8	0.5	10/1.5= 6.67	1	5.57
1.0 X 10^9	1.0	10/1.5= 6.67	1	5.57

3. RESULTS AND DISCUSSION

The up-down approach was utilized for animal welfare reasons and although this method was time-consuming, it did result is fewer animals being sacrificed. In choosing a starting dose, a literature search was conducted which focused on the chosen organisms and infectious studies where these organisms were administered via the intraperitoneal route. Literature indicated that ATCC *Staphylococcus aureus* 29213 had been administered at a dose of 1.0 X 10^{10}cfu/ml to Wistar rats [10] in an attempt to determine dephosphorylation of the endotoxin produced. This dose was therefore chosen as the start dose but when administered, no clinical signs was observed suggesting that the animals had no significant reaction to the organism. The dose was therefore doubled to 2.0 X 10^{10} cfu/ml. All animals in the test group had to be terminated within 6 hours of administration of this dose due to clinical signs such as lethargy, ruffled fur, porphyrin staining, dehydration and hunched back that were observed. On post mortem, there was a lot of gas production in the intestine which gave a decomposed scent. The livers appeared normal but there was slight enlargement of the spleens and discoloration of the kidneys (dark green). It appeared that the animals developed disseminated intravascular coagulation (DIC). A dose of 1.5 X 10^{10} cfu/ml was next administered and the animals showed similar results to those that were administered 1.0 X 10^{10} cfu/ml. In an attempt to carefully titrate the dose, 1.75 X 10^{10} cfu/ml was administered. At this dose, although the animals displayed a number of clinical signs (Table 1), they survived. To titrate the dose to lethality, 1.87 X 10^{10} cfu/ml was administered and all rats succumbed to the infection within 8 hours after the dose was administered. The dose of 1.75 X 10^{10} cfu/ml was therefore selected as the optimum dose for *S aureus*.

Although the animals at the higher doses remained alive for a few hours, we decided against using them as our infective dose because we wanted to mimic a human scenario where a patient would be ill for some time before being admitted to the emergency room. The higher doses resulted in euthanasia much too early in the course of infection. The dose of 1.75 X 10^{10}cfu/ml allowed us to observe the clinical signs of infection (Table 3), and even though the animals survived the infection, there were notable pathological changes (Table 4) observed at ten (10) days post infection. The animals showed labored abdominal breathing and abdominal wasting. There was also signs of increased respiration, porphyrin staining, tremors and vent staining.

The same approach was applied to *Pseudomonas aeruginosa* ATCC 27853. Cirioni et al. [11] used a dose of 2.0 X 10^{10} cfu/ml. It was thought that this was too high a start dose for two main reasons. Firstly, Cirioni et al aimed to induce sepsis in an attempt to determine the role of a Histadin derivative, thus they initiated treatment with antibiotics immediately after infecting the animals. Using such a high dose without and antibiotic will lead to early death of the animals and would defeat the purpose of the

present study. The second reason is that the gram-negative *Pseudomonas aeruginosa* would be more virulent and lethal in terms of infection than *Staphylococcus aureus*. Thus, the starting dose of 0.5 Mc Farland standard was employed for *Pseudomonas aeruginosa*. However, the animals survived the dose with virtually no clinical signs of infection. The does was therefore increased to 1.0 x 10^9 cfu/ml and the animals died about 4-6 hours after infection. On postmortem, however, the animals showed little sign of reaction to the infective agent. It was thought that, perhaps, the animals developed rapid peritonitis and sepsis and died as a result of DIC. A middle dose of 5.0 X 10^8 cfu/ml was next attempted and, again, a similar result to those infected with 1.0 x 10^9 cfu/ml was observed: all the animals succumbed to the infection. In an attempt to titrate the dose as described earlier, a dose of 3.0 X 10^8 cfu/ml was administered and the result then mimicked infection in humans. The animals showed classic clinical signs of infection and half the population survived up to 18-hour post infection. Ideally, the experimental protocol required a dose which would induce the clinical signs of infection, but which the animal would eventually recover from. In the *Pseudomonas aeruginosa* study, therefore, the Lethal Dose (LD_{50}) was attained. At this dose, animals displayed classical signs of infection and those that survived up to18-hourpost-infection, eventually recovered from the infection. The dose chosen resulted in a number of post mortem changes which were indicative of local reaction to the infective agent. The *S. aureus* group was able to start treatment at the 18 hours' post-infection mark. The *P. aeruginosa* proved a challenge as half the population died. The time for initiation of therapy was at the six-hour mark as the animals succumbed to the infection some time later. This was evident as shown in Tables 3a, 3b, 3c and 4. The pictures in Figs. 1-14 show some of the pathological changes which were observed for both study groups.

Table 3a. Clinical signs displayed by Sprague Dawley rats after infection with various doses of *Staphylococcus aureus* (ATCC 29213)

Clinical sign	Group 1	Group 2	Group 3	Group 4	Group 5
	1.0 X 10^{10}	1.5 X 10^{10}	1.75 X 10^{10}	1.87 X 10^{10}	2.0 X 10^{10}
Animal survival @ 24 hours post infection	Yes (4/4)	Yes (4/4)	Yes (4/4)	No (0/4)	No (0/4)
Abdominal breathing	No	No	Yes	Yes	Yes
Abdominal Wasting	No	No	Yes	No	No
Diarrhea	No	No	Yes (3/4)	No	No
Increased respiration	No	No	Yes	Yes	Yes
Lethargy	No	Yes	Yes	Yes	Yes
Loss of righting reflex	No	No	No	No	No
Lying on sides	No	Yes (2/4)	Yes (3/4)	Yes	Yes
Porphyrin staining	No	No	Yes (3/4)	No	No
Tremors	No	No	Yes	Yes	Yes
Vent staining	No	Yes (2/4)	Yes (3/4)	No	No

Table 3b. Clinical signs displayed by Sprague Dawley rats after infection with various doses of *Pseudomonas aeruginosa* (ATCC 28753)

Clinical signs	Group 1	Group 2	Group 3	Group 4
	1.5 X 10^8	3.0 X 10^8	5.0 X 10^8	1.0 X 10^9
Animal survival @ 24 hours post infection	Yes (4/4)	Partial (2/4)	No (0/4)	No (0/4)
Abdominal breathing	No	Yes	Yes	Yes
Abdominal Waist	No	Yes	No	No
Diarrhea	No	No	No	No
Increased respiration	No	Yes	Yes	Yes
Lethargy	No	Yes	Yes	Yes
Loss of righting reflex	No	No	No	No
Lying on sides	No	Yes	Yes	Yes
Porphyrin staining	No	Yes	No	No
Tremors	No	Yes	Yes	Yes
Vent staining	No	Yes	No	No

Table 3c. Post mortem changes observed in Sprague Dawley rats after infection with various doses of *Staphylococcus aureus* (ATCC 29213)

Post mortem changes	Group 1	Group 2	Group 3	Group 4	Group 5
	1.0 X 10^{10}	1.5 X 10^{10}	1.75 X 10^{10}	1.87 X 10^{10}	2.0 X 10^{10}
Animal survival	Yes (4/4)	Yes (4/4)	Yes (4/4)	No (0/4)	No (0/4)
Ascites	No	No	Yes	No	No
Abscesses on intestines	No	Yes	Yes	No	No
Abscess on kidney	No	No	Yes (3/4)	No	No
Abscess on liver	No	Yes (2/4)	Yes	No	No
Abscess on spleen	No	No	Yes	No	No
Adrenals enlarged	No	No	Yes (2/4)	No	No
Fibrin tags	No	Yes	Yes (3/4)	No	No
Increased pericardial effusion	No	No	Yes (3/4)	No	No
Rounded liver		No	Yes	No	No
Splenomegaly	No	No	Yes	No	No

Table 4. Post mortem changes observed in Sprague Dawley rats after infection with various doses of *Pseudomonas aeruginosa* (ATCC 27853)

Post mortem changes	Group 1	Group 2	Group 3	Group 4
	1.5 X 10^{8}	3.0 X 10^{8}	5.0 X 10^{8}	1.0 X 10^{9}
Animal survival	Yes (4/4)	Partial (2/4)	No (0/4)	No (0/4)
Ascites	No	Yes	No	No
Abscesses on intestines	Yes	Yes	No	No
Abscess on kidney	No	Yes (3/4)	No	No
Abscess on liver	No	Yes	No	No
Abscess on spleen	No	Yes	No	No
Adrenals enlarged	No	No	No	No
Fibrin tags	No	Yes	No	No
Increased pericardial effusion	No	No	No	No
Rounded liver	No	Yes	No	No
Splenomegaly	No	Yes (3/4)	No	No

Thus, infective doses of each organism were determined. However, the animals reacted differently to each organism and produced different results in each case. In using the UDP, it was possible to use fewer animals than would have been required to carry out a classic lethal dose study.

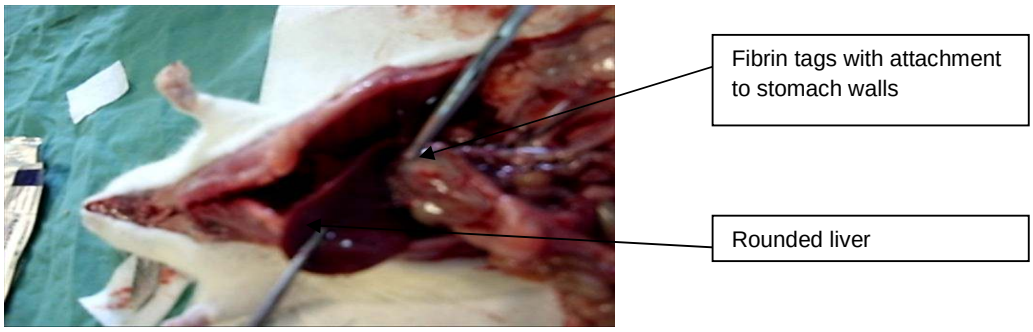

Fig. 1. Post mortem changes at day 10 after IP inoculation of *S. aureus* (1.75 x 10^{10}cfu) showing fibrin tags under liver with attachment to stomach wall

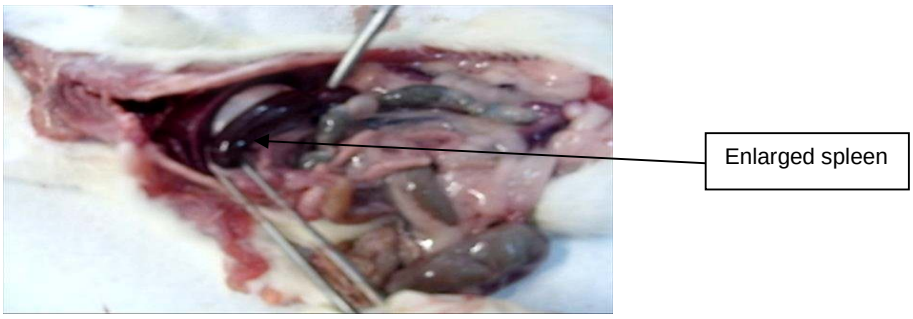

Fig. 2. Post mortem changes at day 10 after IP inoculation of *S. aureus* (1.75 x 10^{10}cfu) showing splenomegaly

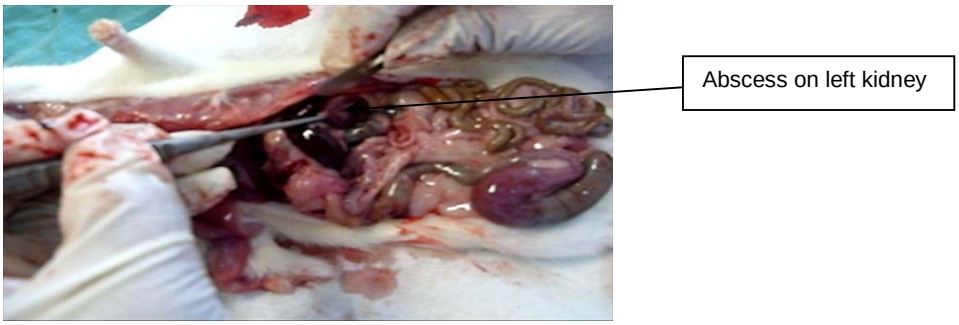

Fig. 3. Post mortem changes at day 10 after IP inoculation of *S. aureus* (1.75 x 10^{10}cfu) showing abscess on left kidney

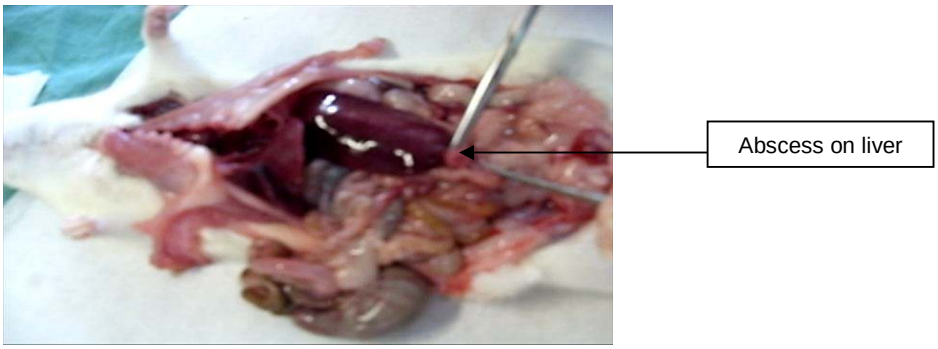

Fig. 4. Post mortem changes at day 10 after IP inoculation of *S. aureus* (1.75 x 10^{10}cfu) showing abscess formation on liver

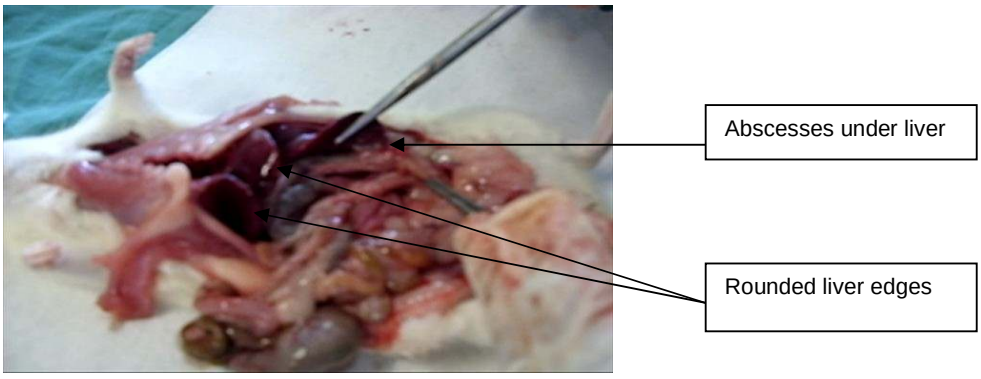

Fig. 5. Post mortem changes at day 10 after IP inoculation of *S. aureus* (1.75 x 10^{10}cfu) showing multiple abscesses under liver and rounded liver edges

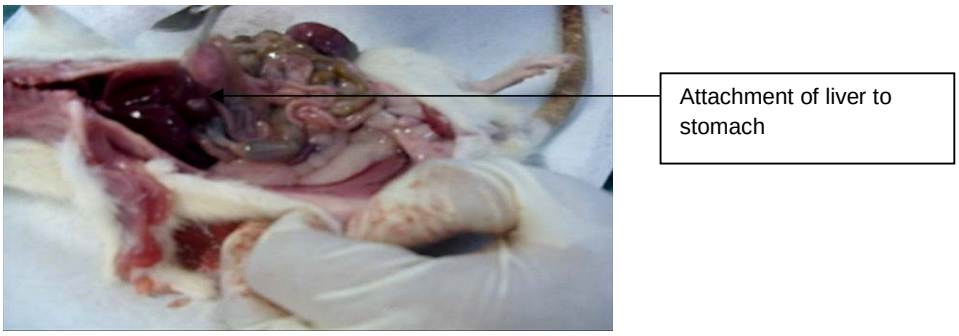

Fig. 6. Post mortem changes at day 10 after IP inoculation of *S. aureus* (1.75 x 10^{10}cfu) showing fibrin tags with liver attachment and adhesions

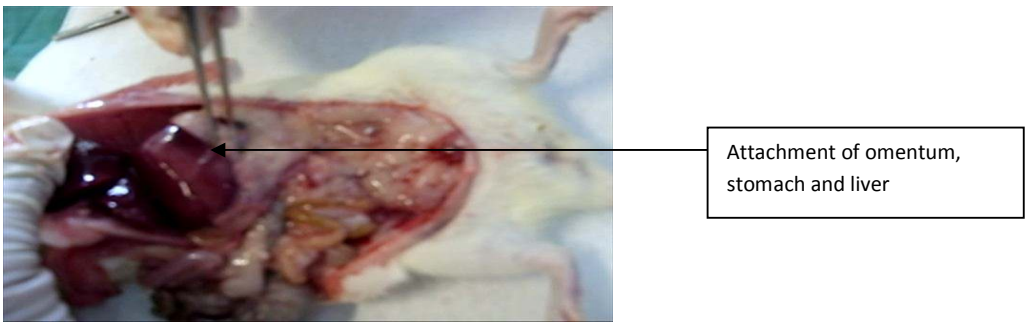

Fig. 7. Post mortem changes at day 10 after IP inoculation of *S. aureus* (1.75 x 10^{10}cfu) showing attachment between omentum, stomach and liver

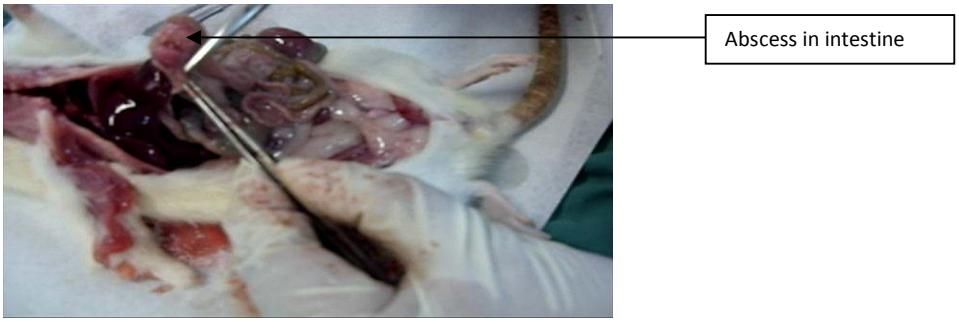

Fig. 8. Post mortem changes at day 10 after IP inoculation of *S. aureus* (1.75 x 10^{10}cfu) showing abscess material in intestine

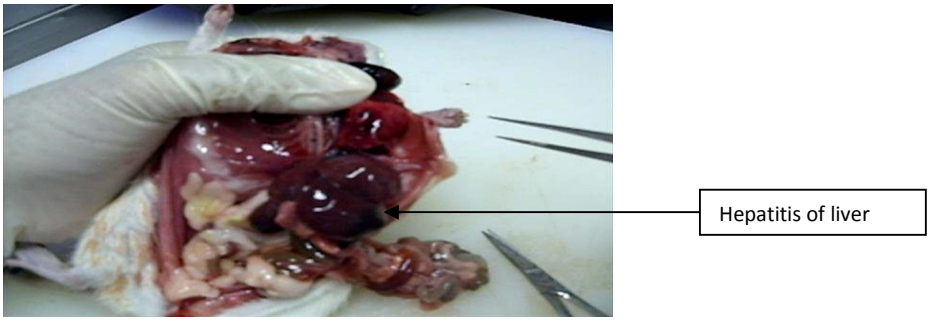

Fig. 9. Post mortem changes at day 10 after IP inoculation of *P. aeruginosa* (3.0 x 10^{8}cfu) showing hepatitis of liver

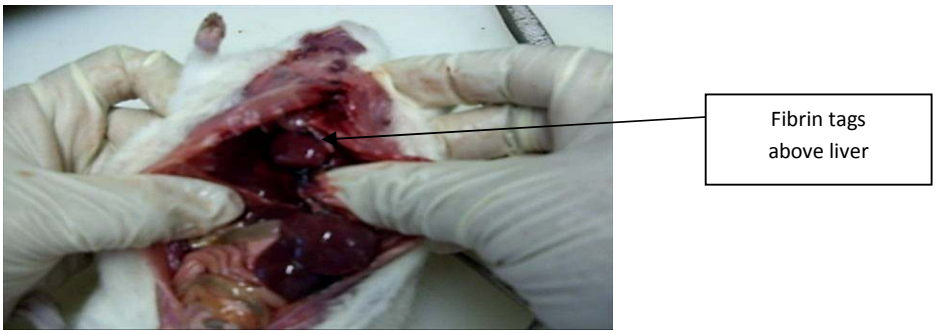

Fig. 10. Post mortem changes at day 10 after IP inoculation of *P. aeruginosa* (3.0 x 10^8cfu) showing fibrin tags above liver

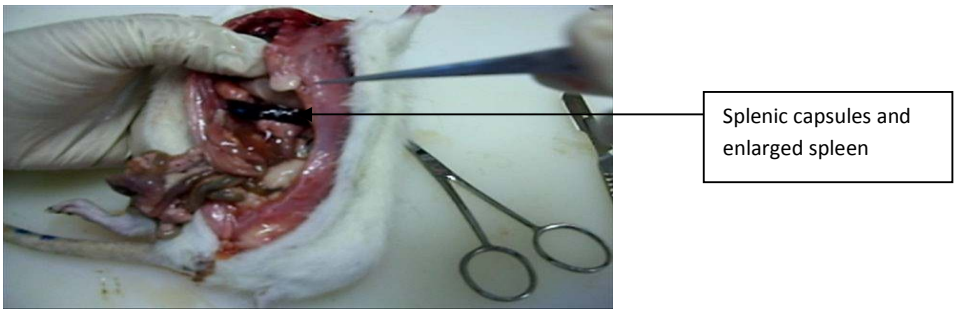

Fig. 11. Post mortem changes at day 10 after IP inoculation of *P. aeruginosa* (3.0 x 10^8cfu) showing splenic capsules and enlarged spleen

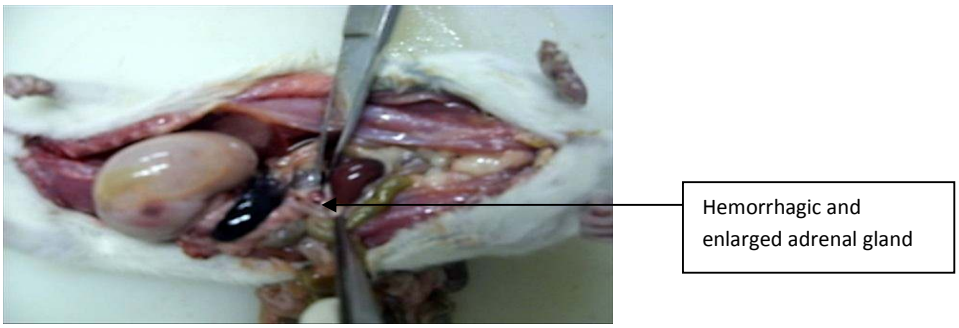

Fig. 12. Post mortem changes at day 10 after IP inoculation of *P. aeruginosa* (3.0 x 10^8cfu) showing adrenal glands which were hemorrhagic and enlarged

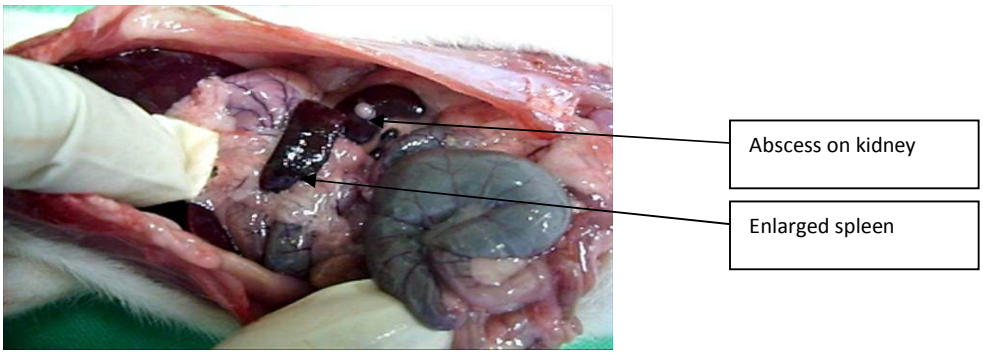

Fig. 13. Post mortem changes at day 10 after IP inoculation of *P. aeruginosa* (3.0 x 10^8cfu) showing splenic capsules, enlarged spleen, abscess on kidney

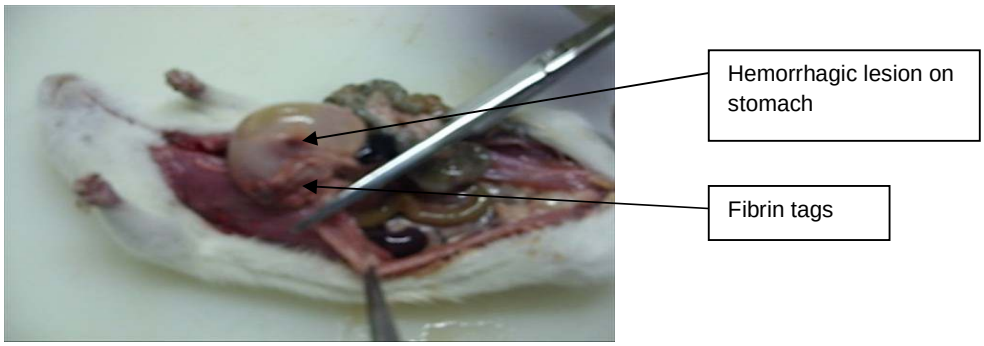

Fig. 14. Post mortem changes at day 10 after IP inoculation of *P. aeruginosa* (3.0 x 10^8cfu) showing hemorrhagic lesion of stomach and fibrin tags

4. CONCLUSION

The UDP proved to be an excellent method for determining infective and/or lethal doses of tested organisms. It allowed examination of individual groups of animal at a time. It also allowed for a reduction in the number of animals needed for the study. From this study, it appears reasonable to assume that the infective dose for *Staphylococcus aureus* (ATCC 29213) is about 1.75 X 10^{10} cfu. This was the highest dose that was tolerated by the animals and even though the animals survived, there were notable clinical signs of infection as well as pathological changes involving the liver, kidney and spleen. With respect to the gram-negative *Pseudomonas aeruginosa* (ATCC 27853), the dose of about 3.0 x 10^8 cfu seems to be the LD_{50} for the organism. Half of the population died and those that survived displayed similar signs of infection and gross pathological changes as the animals infected with *S. aureus*.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Department of Graduate Studies, University of the West Indies, St Augustine. The application for animal research was approved by the Animal Ethics Committee, Faculty of Medical Sciences, UWI. All experiments have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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