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HAEMATOLOGICAL EVALUATION OF SPRAGUE-DAWLEY RATS INFECTED WITH *PASTEURELLA MULTOCIDA* AND ADMINISTERED *BDELLOVIBRIO BACTERIOVORUS* (ATCC™ 1534) AS THERAPY

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ABSTRACT

The effects on haematological parameters of Sprague-Dawley rats infected (challenged) by the injection of pathogenic *Pasteurella multocida* and the administration of *Bdellovibrio bacteriovorus* (ATCC™ 1534) as therapy, occasioned by the consideration of its use as an alternative to antibiotics, due to the high rate of bacterial resistance to current clinically used antibiotics was investigated. A total of 60 rats, divided into 5 groups (4 experimental groups and 1 control group) of 12 rats each were used in this study. The first group of 12 rats were injected subcutaneously with one millilitre of 1 x 10^8/ml *B. bacteriovorus* (ATCC™ 1534). The second group of 12 rats were injected with one millilitre 10^8/ml of *P. multocida* in saline. One millilitre each of 10^8/ml of both *P. multocida* and *B. bacteriovorus* (ATCC™ 1534) were injected into another set of 12 rats in the third group. The third group of 12 rats were injected once intra-muscularly, in the hind flank muscle, with 2 mg/kg of Ketamine Hydrochloride. And lastly, one set of 12 rats were not injected with any bacteria served as control. In all cases, observed haematological data were analysed from the experimental rats after 168 hours (except rats which were injected with 2mg/kg ketamine hydrochloride, used as anaesthetic, (at sacrifice), and compared with the haematological profiles of the 12 control rats. Results shows a reduction of mortality from 9 to 1 (88.8%) of rats challenged with *P. multocida* over those inoculated with *B. bacteriovorus* and *P. multocida* was observed. WBC counts were higher (4.12 x 10^3/µL) in *B. bacteriovorus* and *P. multocida* over WBC counts in control rats which served as WBC reference values. Though, not statistically significant (ANOVA = $p > 0.05$). In a similar comparison, RBC counts (6.5 x 10^3/µL) were lower than observed in control rats, while platelet counts (1138 x 10^3/µL) were higher than values in controls, however, this was statistically significant. Moreover, haemoglobin concentrations were lower (11.7 g/dL) than in control rats. Though there were slight variations in haematological profiles from reference values, it was concluded that *B. bacteriovorus* seems to have no life-threatening effect on haematology of rats. However, evaluations such as observed platelet increases on inoculations of *B. bacteriovorus*, need to be addressed before the promise of its *in vivo* use in controlling Gram-negative infection in animals and humans can be tapped.

Keywords: *bdellovibrio bacteriovorus, control, haematological, Pasteurella multocida, rats*

INTRODUCTION

Bdellovibrio are Gram-negative, vibroid and tiny bacteria (between 0.3 - 0.5μm by 1.4 - 2.5μm). They are motile by single sheathed polar flagella. They are predatory on other Gram-negative bacteria. Using degradative enzymes, they create pores in host cell walls, enter the periplasm, and use the cytoplasmic contents of prey cells as nutrients for growth and reproduction. Finally, they burst the host cell envelopes, which lead to the host cell (prey) deaths (Megan *et al*., 2003).

Bdellovibrio interest microbiologists as it has potential for use as bio-controls of many pathogenic Gram-negative microorganisms. In addition, studies on enzymes they use to degrade host cell walls give insight into targets in prey cells that have been successful points of attack from an evolutionary viewpoint and can be used for design of antimicrobial agents (Lambert *et al.*, 2006).

Though, other predatory bacteria e.g. *Micavibrio,* are also being studied for *in vivo* predatory activity, *Bdellovibrio* stands out because of a number of unique properties, such as having a wide variety of Gram-negative prey, which is a highly desirable quality of a good microbial control agent. *Bdellovibrio* have been isolated from water bodies and soils from parts of Benue State, Nigeria (Sar *et al*., 2015; Sar *et al*., 2016), and from the gastro-intestinal tracts of animals and humans (Houmsou *et al*., 2010). Soon, *Bdellovibrio* may have practical antimicrobial therapeutic applications in medical science (Harini *et al*., 2013).

MATERIALS AND METHODS

A total of 60 (30 of either sex) randomly selected Sprague Dawley rats, approximately 16 weeks old were used. They were housed under ambient temperatures of about 25 ^{o}C ± 5 ^{o}C, and relative humidity (RH) of about 55% ± 5%. Feed and water were supplied *ad libitum.*

All rats used were from the same litter thus, genetically related, at similar stages of development, and received similar treatments, such that within limits, haematological parameters from them would originally have insignificant variations, such that any major changes or shifts observed should be due to experimental treatments.

To determine the haematological effects on rats, a group of 12 rats were each subcutaneously (abdominally) injected with 1 ml 10^8/ml *Bdellovibrio bacteriovorus* (ATCC 15364) suspension. Further, to determine similar effects of *Pasteurella multocida*, another set of 12 rats were injected with one millilitre of 10^8/ml suspension of *P. multocida*. For investigating *in vivo* effects of *B. bacteriovorus* on *P. multocida*, another set of 12 rats were injected first with *P. multocida,* then within a minute or two, injected with 1 ml of 1 x 10^8 PFU/ml *B. bacteriovorus* (ATCC 1534) suspension.

To evaluate the haematological effects of Ketamine Hydrochloride, an anaesthetic used on the rats during blood collection and sacrifice, a control group of 12 rats were injected once intra-muscularly, in the hind flank muscle, with 2 mg/kg of Ketamine Hydrochloride. These rats were sacrificed as soon as the drug took effect. A second control group was made up of 12 rats not injected with pathogen, *B. bacteriovorus* or anaesthetic.

Apart from anaesthetic, all other injections were subcutaneous – a fold of abdominal skin was pinched and lifted for each injection. All injections were given once every 24 hours, for 168 hours, after which period the rats were bled (fur over the sternum was thoroughly cleaned with tincture of iodine, and one millilitre of blood withdrawn

by cardiac puncture) for haematological analysis. All injected rats were observed for pathological, physiological and physical signs of disease, including mortality.

DETERMINATION OF BACTERIAL PATHOGENICITY

P. multocida was serially diluted in physiological saline. The count of the bacteria in each dilution was determined by McFarland's standard. Six animals each were subcutaneously injected with one dilution of inocula. Mortality was considered a sign of infection. Live rats were sacrificed at 168 hours for gross anatomical lesions/histology, and the LD_{50} calculated according to the method of Reed-Muench (Saganuwan, 2015).

BACTERIAL COUNTS DETERMINATION

SD 2303 McFarland Standard 3.0 and the test bacterial suspension were employed using model WPA CO 7500 Colorimeter. The McFarland Equivalence Standards was used to estimate bacterial quantities in suspensions. These produced expected bacterial plate counts and were used for the tests.

Further, bacterial counts obtained by McFarland's standard were run through Invitrogen™ Countess™ (USA) Automated Cell Counter (ACC). Numbers of viable cells were automatically obtained and recorded.

AUTO-ANALYSIS OF BLOOD PARAMETERS

The analysis was carried out by Mindray™ model BC 5300 (China) auto-haematology analyzer. Parameters determined were red blood cell (RBCs), platelet (PLT), white blood cell (WBC) counts and haemoglobin (HGB) concentration.

STATISTICAL ANALYSIS

Data were analyzed using IBM SPSS version 23.0 (2015). ANOVA and *post hoc* multiple comparisons were used to test for group differences between haematological parameters. All tests were considered significant at $p \leq 0.05$ level of probability.

RESULTS

MORTALITY RATES

A total mortality rate of 75% (n=9; 5 male and 4 female) was observed in rats injected with *P. multocida,* and 8.3% (n=1 female rat) in those injected with both *P. multocida* and *B. bacteriovorus* (Figure 1). Twenty-four hours after injection with *P. multocida*, there was a mortality rate of 41.7% (5 rats). After 96 hours (4 days) of injections, there was a mortality rate of 66.7% (8 rats), which rose to 75% by 168 hours of injection. After injecting rats with both *P. multocida* and *B. bacteriovorus* however, mortality fell by 88.8% compared with rats injected with only *P. multocida*.

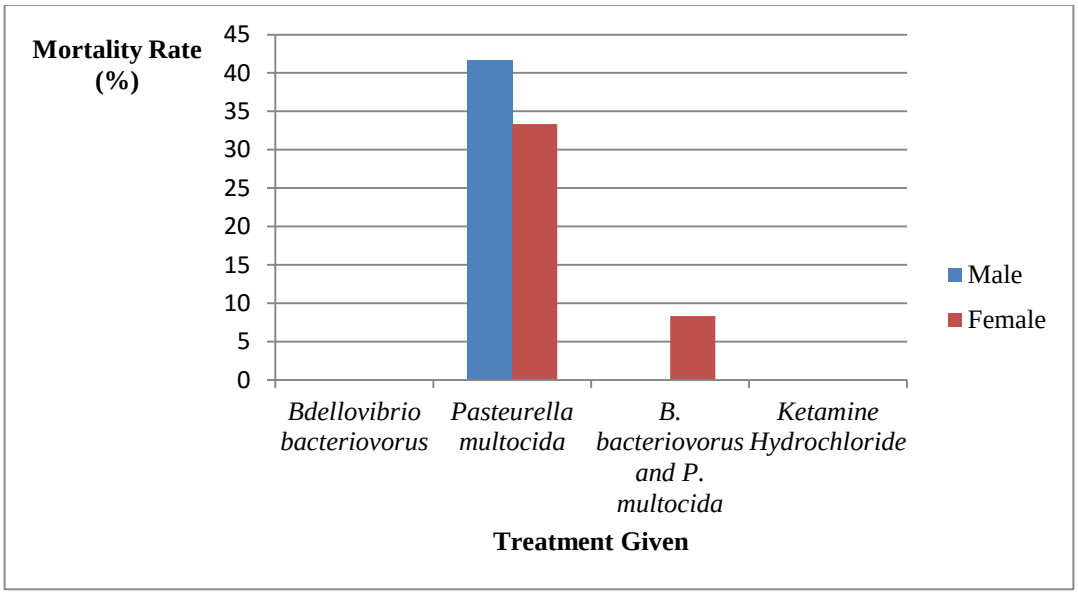

Figure 1: Mortality Rates in Sprague Dawley Rates Injected with *B. bacteriovorus*, and with *P. multocida* and *B. bacteriovorus* + *P. multocida*

CHANGES IN HAEMATOLOGICAL PARAMETERS

WHITE BLOOD CELL (WBC) COUNTS

Table I shows changes in haematological parameters in rats injected with *B. bacteriovorus*, with *P. multocida* and with both *P. multocida* and *B. bacteriovorus*. *P. multocida* injected rats had a WBC count of 7.50 x 10^3/µL, while rats injected with both *B. bacteriovorus* and *P. multocida* had a value of 4.12 x 10^3/µL, indicating a lower count of 3.38 x 10^3/µL or 45.1%. However, no statistically significant difference in WBC counts was found between rats injected with only *B. bacteriovorus* and control rats not injected.

RED BLOOD CELL (RBC) COUNTS

When injected with *P. multocida*, RBC counts (7.00 x 10^6/µL) were higher compared with counts (6.50 x 10^6/µL) in rats injected with both *B. bacteriovorus* and *P. multocida* (Table I) indicating a decrease of 0.50 x 10^6/µL (7.14%). Compared with control rat's RBC values, all injected rats had lower counts. Statistically, RBC counts of rats injected with *B. bacteriovorus* were not significantly different from un-injected control rats. Similarly, RBC counts between rats injected with *P. multocida* only and the un-injected controls were significantly different. Again, RBC counts differed significantly between rats injected with only *P. multocida*, and rats injected with both *P. multocida* and *B. bacteriovorus*.

PLATELET (PLT) COUNTS

P. multocida injected rats had a platelet count of 1004 x 10^3/µL, which increased to 1138 x 10^3/µL in the *B. bacteriovorus* and *P. multocida* injected rats, indicating an increase of 134 x 10^3/µL (13.4%). Both groups showed counts higher than control rat counts (Table I). PLT counts differed significantly between rats injected with *B.*

bacteriovorus and un-injected controls. Similarly, PLT counts differed significantly between rats injected with *P. multocida,* and rats injected with both *P. multocida* and *B. bacteriovorus* and un-injected controls.

HAEMOGLOBIN (HGB) CONCENTRATION

P. multocida injected rats had a HGB concentration of 12.10 g/dL, which decreased to 11.70 g/dL in the *Bdellovibrio* and *P. multocida,* treated rats, a decrease of 0.40 g/dL (3.31%) (Table I). Haemoglobin concentration in rats injected with only *B. bacteriovorus* was significantly different from un-injected controls. HGB concentration also differed significantly between rats injected with only *P. multocida* and those injected with both *P. multocida* and *B. bacteriovorus.* Similarly, significant difference in HGB concentration was found in rats injected with *P. multocida* and un-injected control rats.

Table I: Haematological changes in rats injected with *P. multocida,* and with *P. multocida* and *B. bacteriovorus*

Organism Injected *(Groups)*	Parameter Analysed							
	WBC *(x 10^3/µL)*	*SD*	RBC *(x 10^6/µL)*	*SD*	PLT *(x 10^6/µL)*	*SD*	HGB *(g/dL)*	*SD*
B. bacteriovorus	5.52	±3.5	7.31	±0.6	818	±292.3	13.0	±1.04
P. multocida (a)	7.50	±0.7	7.0	±0.09	1004	±69.2	12.1	±0.11
P. multocida + *B. bacteriovorus (b)*	4.12	±1.4	6.5	±1.1	1138	±366.8	11.7	±1.5
Difference *(a-b)*	3.3	±1.72	0.5	±0.3	-134	±67.0	-0.4	±0.2
Percent Change	45.1	-	7.14	-	13.4	-	3.31	-
**Reference Value*	*3.82*	-	*8.21*	-	*916*	-	*16*	-

Key: *N* = 12. WBC =White Blood Cells; RBC = Red Blood Cells; PLT = Platelets; HGB = Haemoglobin; *SD* = Standard Deviation; - = Not Applicable; *Un-injected Control Rats

DISCUSSION

An interesting result of the mitigating effects of *Bdellovibrio* on *P. multocida* infection was the sharp drop in mortality of rats injected with both *P. multocida* and *B. bacteriovorus. In vivo* predation of pathogen by *B. bacteriovorus* could have accounted for a reduction in mortality rates. In a similar finding, Willis *et al.* (2016) injected Zebrafish larvae with *Shigella flexneri* alongside *B. bacteriovorus* and found that *Bdellovibrio* effectively predated *in vivo* on *S. flexneri* in the Zebrafish host, leading to reduced pathogen concentration and a drop in host mortality. Hobley *et al*. (2006) have also demonstrated by mathematical modelling that *Bdellovibrio* is capable of effective *in vivo* predation in the presence of cellular debris and decoys within living systems.

Higher WBC counts in rats injected with *P. multocida* than in those injected with only *B. bacteriovorus*, and those injected with both *B. bacteriovorus* and *P. multocida*, which counts were all higher than in control un-injected

rats are not isolated findings but indicate that WBCs, which typically increase in numbers on challenge with certain threshold quantities of foreign immunogenic materials, such as pathogens or other foreign substances, may have been triggered by the injected bacteria. Studies by Monnappa *et al*. (2016) showed that *B. bacteriovorus* and other predatory bacteria elicit mild immunologic responses in animals. If this is the case, then injecting rats with *B. bacteriovorus* would have led to the observed rise in WBC levels. From this premise, it follows therefore that injections of both *B. bacteriovorus* and pathogen would have produced an even greater synergistic response occasioned by the sum of the separate immunologic responses to both *P. multocida* and *B. bacteriovorus* injection.

Shatzkes *et al*. (2016) evaluated the ability of predatory bacteria in attenuating *Klebsiella pneumoniae* burden in rat lungs and found that the predatory bacteria reduced pathogen burden and caused transient elevated levels of inflammatory cytokines. It is therefore not unusual to have found a WBC increase in rats injected with *B. bacteriovorus*. Furthermore, work by Willis *et al.* (2016) also found that *B. bacteriovorus* injected into Zebrafish larvae was eventually eliminated by host neutrophils and macrophages, which recognised and engulfed the predator. All these point to the fact that *Bdellovibrio* causes immune responses in animal host as also found in this study.

B. bacteriovorus elicited increase of WBC counts could be an added advantage in that the higher numbers of circulating WBC would help in warding off bacterial infections (Willis *et al*., 2016). Successful elimination of pathogens, even if not directly due to predation by *B. bacteriovorus*, would be more desirable than use of chemotherapeutic alternatives with the attendant unpleasant side effects and adverse reactions frequently associated with their use.

While a plausible explanation for the observation that *B. bacteriovorus* injection lowered RBC counts in rats is hard to come by, it is likely that the low RBC counts may be related to the high WBC counts which could have led to anaemia or even a leukaemia-like situation, which could be harmful (Merchant *et al*., 2004).

An implication of the observation that rats injected with only *Bdellovibrio* had lower platelet counts than control rats is that clotting may be compromised and periods of bleeding during injury increased in recipients of *B. bacteriovorus* therapy. It is possible that certain undetermined factors, components or substances produced by *Bdellovibrio* were released into the bloodstream and could have led to a depletion of platelets. In this study, the presence of such components was not investigated, and remains to be determined.

However, the higher platelet counts in rats injected with both *B. bacteriovorus* and *P. multocida* than found in controls may reflect as in similar findings which observed that platelet counts in humans' increase on infections or certain diseases (Hsu *et al*., 2010). Therefore, inoculating rats with both the *B. bacteriovorus* and *P. multocida* probably led to the high platelet counts observed.

The consistently lower haemoglobin concentrations in rats injected with *P. multocida* and with combinations of *B. bacteriovorus* and *P. multocida* than in un-injected controls may be related to the low RBC counts, as haemoglobin in all mammals is transported by the RBCs. Also, according to Marković *et al.* (2011) several substances may affect rat haematologic parameters, however, the exact mechanism which caused lowered haemoglobin levels, observed in this study, remains unclear.

CONCLUSION

It was shown that *B. bacteriovorus* administered to rats challenged with a pathogenic Gram-negative bacterium, *P. multocida* led to a sharp reduction in their mortality rates, which suggests that the organism mitigated the pathogen's morbid effects. However, certain effects associated with injections of *B. bacteriovorus*, such as differences in WBC, reduction in RBC counts and haemoglobin concentration, as well as increased platelet counts, though mild and perhaps not clinically important requires further investigation and should be fully accounted for, to prepare grounds for eventual use *Bdellovibrio* for treatment of Gram-negative bacterial infections in animals.

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