JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

VIRULENCE STUDIES OF *PASTEURELLA MULTOCIDA* IN MICE, DUCKLINGS AND ADULT DUCKS

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doi: 10.15414/jmbfs.2017.6.5.1209-1212

ARTICLE INFO

Received 14. 10. 2013
Revised 26. 2. 2017
Accepted 6. 3. 2017
Published 3. 4. 2017

Regular article

OPEN ACCESS

ABSTRACT

Pasteurella multocida is a Gram negative bacterium causing severe disease in a multitude of hosts; the A: 1 strain of this bacterium is extremely virulent to hosts. In this study, the virulence of *P. multocida* A: 1 strain was assessed in one month old ducklings, six months old ducks and eight weeks old albino mice. The groups of experimental animals were inoculated with the different dilutions of the bacteria through subcutaneous route. The control groups were sham inoculated with sterile phosphate buffered saline via respective routes. Mortality was recorded for two weeks post inoculation. All the dead animals and birds were examined for specific gross lesions of pasteurellosis and attempted re-isolation of the organism on blood agar from their internal organs. The median lethal dose was calculated using Reed and Muench method. For ducklings, the median lethal dose was 13 colony forming units and the dilution giving 50 % end point with 0.1 ml subcutaneous dose was $10^{-7.4}$. The median lethal dose could not be arrived at for mice and adult ducks as the strain killed all the inoculated mice even in the lowest dilution tested, while most of the adult ducks were resistant to infection.

Keywords: *Pasteurella multocida*, Lethal dose, Virulence, Duckling, Mice, Gross pathology, Lesion score

INTRODUCTION

Pasteurella multocida (P. multocida), the Gram negative bacterium is responsible for numerous economically significant diseases in a variety of hosts like cattle, buffalo, sheep, goat, domestic fowl, turkey, horse, camel, wild animals and wild birds (**Snipes *et al*., 1988; Boyce *et al*., 2004**). Pasteurellosis is a persistent problem in developing countries especially in South East Asia and Africa. *P. multocida* have many serotypes, of which Serotype A is mainly responsible for causing Fowl Cholera or Avian pasteurellosis.

Several virulence studies had been conducted by many researchers to quantify the virulence of *Pasteurella* through various routes (**Rimler and Glisson, 1997**). All the studies echo one thing that the virulence of bacteria is complex, multi-factorial and it can vary depending on the strain used, host studied, route, dose of administration and many other unknown confounding variables. Virulence of a pathogen is measured in suitable experimental animals, the end result of which is death. Virulence testing forms an integral part of vaccinology and pharmacology, especially for computing the challenge dose during potency testing of vaccines and various drugs. Fifty per cent lethal dose or LD_{50} is defined as the number of bacteria required to kill half of the exposed hosts and is shown to be a practical and reliable measure of pathogenicity (**Thomas and Elkinton, 2004**).

The study was done to assess the virulence of avian origin *Pasteurella multocida* A: 1 strain in three different animal host models (1) one month old ducklings (2) six months old ducks and (3) eight weeks old albino mice.

MATERIALS AND METHODS

Bacterial strain

The *P. multocida* A: 1 strain was isolated from a very severe outbreak of pasteurellosis at Niranam Duck farm, Pathanamthitta District, Kerala State, India. The isolate was biochemically identified and serotyped as *P. multocida* serotype A, biovar 1 at Indian Veterinary Research Institute (IVRI), Izatnagar, India. The isolate was named as DP1 and maintained in freeze dried form at the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences (COVAS), Mannuthy, Kerala, India.

Experimental animals

Unvaccinated one month old ducklings (n=54) (*Anas platyrhynchos*, Kuttanad variety) and 6 months old Kuttanad ducks (n=54) were procured from a private breeder at Thrissur, Kerala, India. Swiss albino female mice (*Mus musculus*), eight weeks of age (n=54) were procured from the Small Animal Breeding Station, COVAS, Mannuthy. The ducklings were randomly assigned into nine groups, with six ducklings in each group. The ducks and mice were also similarly arranged into nine groups. The first eight groups served as the test groups while the ninth group served as control group, in all the three trials. Each group of birds was housed separately in locally made isolator cages (of dimensions 6×3×3 cubic feet) which were arranged in two adjacent rooms (4 cages in each room). The control birds were kept in a separate room in order to avoid any chances of cross contamination. Each cage had separate waterers and feeders and the floor being covered with saw dust as litter material. The birds were provided with commercial duck feed (formulated at University Poultry Farm, COVAS) and water *ad libitum*. The groups of mice were kept in plastic shoe box cages and provided with commercial mice feed (formulated at Small Animal Breeding Station, COVAS) and water *ad libitum*. The animals were housed in their respective cages one week prior to the beginning of the experiment for acclimatization. The animals and birds were observed for signs of any disease before the start of experiment.

All the animal experiments were performed with the prior approval of the Institutional Animal Ethics Committee (IAEC) of COVAS, Mannuthy, which follows the guidelines laid by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Median lethal dose (LD_{50}) testing

For determination of LD_{50}, the freeze dried *P. multocida* A: 1 was reconstituted in 0.1 ml Tryptone soya broth (Himedia, Mumbai, India) and cultured on to dextrose starch agar (DSA) (Himedia, Mumbai, India) at 37°C for 24 h. The growth on DSA was harvested, washed thrice in phosphate buffered saline (PBS, pH 7.4) by centrifugation at 3000 × g for 15 min and re-suspended in the same buffer to contain 3×10^9 CFU ml^{-1} using McFarland standards (600 nm). Then

serial tenfold dilutions were made which were quantitatively assessed by plate counting retrospectively.

The test groups of ducklings were inoculated with the prepared dilutions of the bacteria at the rate of 0.1 ml per bird subcutaneously at wing web region. The adult ducks were also inoculated via wing web route but with an increased dose of 0.5 ml. The first eight groups of mice were inoculated with the dilutions, 0.1 ml per mice, intraperitoneally. The control groups were sham inoculated with corresponding volumes of sterile PBS (pH 7.4) via respective routes. Mortality was recorded up to two weeks post inoculation (PI).

All the dead birds and animals were examined for specific gross lesions caused by *P. multocida* and attempted re-isolation of the organism on blood agar from heart blood, liver and spleen under 5-10 % carbon dioxide tension. The live birds and animals after 14 days PI were euthanized and bacteriologically examined. Blood smears and organ impression smears (spleen and liver) were also examined following Leishmans staining.

The method described by **Reed and Muench (1938)** was used for LD_{50} calculation from the recorded cumulative mortality rates. Median lethal dose was calculated from the proportionate distance (PD) using the formula listed below.

The calculation of median lethal dose from PD is as follows,

$$PD = \frac{\text{(\% mortality at dilution next above 50 \%) - 50 \%}}{\text{(\% mortality at dilution next above 50 \%) - (\% mortality at dilution next below 50 \%)}}$$

Log LD_{50} = (logarithm of dilution next above 50 % mortality + (PD × log dilution factor)

Lesion scoring

To assess the lesions, a comprehensive post mortem examination was performed on each bird and the lesions were scored (0-3) for six organs including intestine, lungs, pericardium, heart, liver and spleen as per the criteria listed in Table 1. The same personnel scored the lesions of all dead birds to bring down the error while judging the lesions and care was taken to do the scoring under sunlight. Statistical analyses for lesion scores were conducted using one way Analysis of Variance. Duncan's multiple range test was used to compare means (Duncan, 1955).

Table 1 Scoring system for lesions induced by *P. multocida* in different organs

Organ	Score	Description of lesion
Intestine	0	No lesions
	1	Catarrhal enteritis
	2	Moderate enteritis with petechial haemorrhage
	3	Extensive serosal and mucosal haemorrhage
Lung	0	No lesions
	1	Congestion of lungs
	2	Pulmonary oedema
	3	Pneumonic lungs
Pericardium	0	No lesions
	1	Pericardial effusion
	2	Translucent pericardium, moderate pericarditis
	3	Opaque pericardium, severe pericarditis
Liver	0	No lesions
	1	Congestion of liver
	2	Pin point necrosis, hepatomegaly
	3	Extensively necrosed and pale liver
Spleen	0	No lesions
	1	Congestion of spleen
	2	Mild necrosis and spleenomegaly
	3	Mottling /haemorrahge in spleen
Heart	0	No lesions
	1	Hypertrophy of heart/slight haemorrhage
	2	Moderate haemorrhage on epicardium
	3	Severe epicardial and endocardial haemorrhages

RESULTS

Median lethal dose in ducklings

The dilution giving 50 % end point with 0.1 ml subcutaneous dose was $10^{-7.36}$. The LD_{50} of the isolate was 13 colony forming units (CFU) when tested in one month old ducklings. The percentage of animals dead and alive in each group of ducklings is furnished in Table 2 and the detailed calculations are presented in legend section of Table 2.

The gross lesions observed in experimentally infected ducklings were haemorrhages on epicardium, serous yellow fluid in pericardium (Fig. 1A) pin point and extensive necrosis of liver (Fig. 1B), pin point and echymotic haemorrhages in intestinal serosa and mucosa (Fig. 1C), necrosis and petechial haemorrhage in spleen and pulmonary oedema. The gross pathological lesions observed in different groups of inoculated ducklings and the lesion scores are presented in Table 3. Bacteriological examination revealed bipolar organisms from blood and organ impression smears and colonies suggestive of *P. multocida* from internal organs of all the succumbed ducklings. The control birds did not reveal any bacterial growth following organ culture.

Table 2 Median lethal dose of DP1 in one month old ducklings

Group	CFU per 0.1 ml inoculum	No: of birds tested	No: died	No: alive	Cumulative*			Mortality rate	% mortality
					Dead	Alive	Total		
1	2.9×10^{7}	6	6	0	41	0	41	41/41	100
2	2.9×10^{6}	6	6	0	35	0	35	35/35	100
3	2.9×10^{5}	6	6	0	29	0	29	29/29	100
4	2.9×10^{4}	6	6	0	23	0	23	23/23	100
5	2.9×10^{3}	6	6	0	17	0	17	17/17	100
6	2.9×10^{2}	6	6	0	11	0	11	11/11	100
7	2.9×10^{1}	6	4	2	5	2	7	5/7	71.4
8	2.9×10^{0}	6	1	5	1	7	8	1/8	12.5

Legend: *Cumulative total value for dead and live birds was obtained by adding in the direction of lowest to the highest values (shown by solid arrows).

Table 3 Lesion score of the ducklings post inoculation with DP1

Group	Intestine**	Lungs**	Pericardium**	Heart **	Liver**	Spleen*
1	2.67 ± 0.82^{a}	1.83 ± 0.41^{a}	1.33 ± 0.52^{ab}	3.00 ± 0.00^{a}	2.17 ± 0.75^{a}	0.50 ± 0.55^{a}
2	2.33 ± 0.52^{a}	1.50 ± 0.55^{ab}	0.50 ± 0.55^{cd}	2.83 ± 0.41^{ab}	3.00 ± 0.00^{b}	1.50 ± 1.38^{ab}
3	2.67 ± 0.52^{a}	1.17 ± 0.75^{abc}	1.83 ± 0.41^{a}	2.50 ± 0.55^{ab}	2.00 ± 0.89^{a}	2.00 ± 1.10^{b}
4	2.17 ± 0.41^{a}	1.33 ± 0.52^{ab}	0.67 ± 0.52^{bcd}	2.83 ± 0.41^{ab}	2.17 ± 0.41^{a}	0.83 ± 0.41^{a}
5	2.83 ± 0.41^{a}	1.00 ± 0.63^{bcd}	1.17 ± 0.98^{abc}	2.33 ± 0.52^{b}	1.67 ± 0.82^{a}	0.50 ± 0.55^{a}
6	2.83 ± 0.41^{a}	0.33 ± 0.52^{d}	0.33 ± 0.52^{d}	1.17 ± 0.41^{c}	3.00 ± 0.00^{b}	1.00 ± 0.63^{ab}
7	1.50 ± 0.58^{b}	0.50 ± 0.58^{cd}	0.25 ± 0.50^{d}	1.50 ± 0.58^{c}	1.50 ± 0.58^{a}	0.50 ± 0.58^{a}

Legend: Values expressed as mean ± standard deviation (for groups 1-6, n=6 and for group 7, n=4)
Letters with different superscript within a column differ significantly. ** ($P \leq 0.01$) and *($P \leq 0.05$).

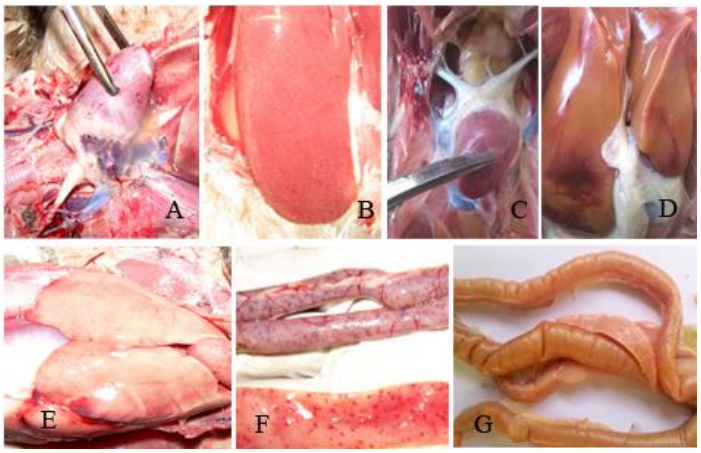

Figure 1 Gross lesions in ducklings following inoculation with *P. multocida* (A) Epicardial petechiae and serous pericardial effusion (lesion score 1) (B) Liver of duckling showing pin point necrosis (diffuse white spots) (lesion score 2) (C) Normal heart from a control bird (D) Liver from control bird showing no gross lesions (E) Extensively necrosed and pale liver (lesion score 3) (F) Multiple haemorrhagic spots in intestinal serosa and mucosa of dead ducklings (lesion score 3) (G) Intestine of control bird without any gross lesion.

Lethal dose 50 in adult ducks

Only one duck died, which was inoculated with 3×10^8 CFU/ml subcutaneously. The other ducks did not succumb and hence it was not possible to arrive at the median lethal dose. The dead duck revealed similar gross lesions as those observed in the case of ducklings. Additionally, extensive petechiation of the peritoneum was noticed (Fig. 2). Despite the successful isolation of the organism from the dead duck, none of the live birds from test groups revealed any of the gross lesions. No growth was observed for live birds on the media following organ culture. The inoculated birds showed some initial symptoms of dullness and drooping which subsided after one day post-inoculation.

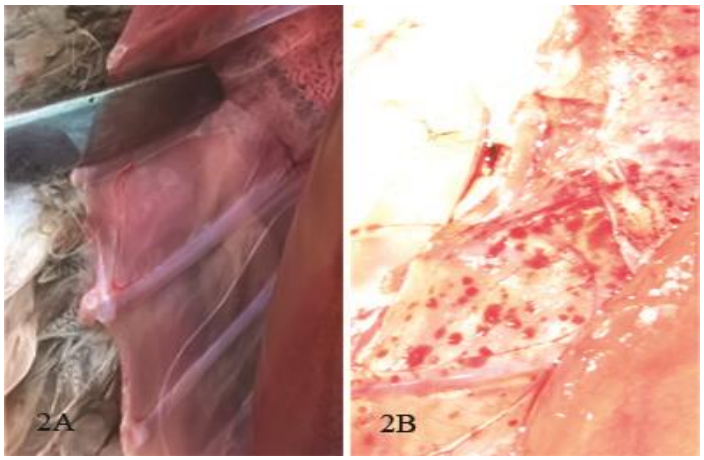

Figure 2 Extensive necrosis of liver and petechiae in peritoneal cavity of ducks experimentally infected with *P. multocida* (2B) compared to no gross lesions in control birds (2A).

Lethal dose in mice

Pasteurella multocida A: 1 strain killed all the inoculated mice within 2 days, even in lower dilutions, while all the mice in the control group remained unaffected. Because of the extreme pathogenicity of the bacteria in mice, the median lethal dose could not be obtained. The gross lesions observed in the inoculated mice were fluid accumulation in peritoneal cavity, petechial haemorrhages in the epicardium, pulmonary oedema and general congestion of all the visceral organs. It was interesting to note that the mice consistently showed peritoneal effusions unlike ducklings and the severe intestinal lesions shown by ducklings were not consistent in mice. Bipolar organisms were detected from blood smears, organ impression smears and the organism was successfully isolated from the internal organs of dead mice. The euthanized control mice revealed no gross lesions and no organisms isolated.

DISCUSSION

Pasteurella multocida possess a multitude of specific virulence factors like hyaluronic acid capsule, lipopolysaccharides, iron regulated outer membrane proteins (IROMPs) etc. This makes the host-bacterium interaction very complex and the virulence depends on the bacterial strain being studied, host model used and many other events occurring *in vivo*, most of which are yet to be elucidated. The gross lesions observed in ducklings were in agreement with **Mbuthia *et al*. (2008)** and **Shilpa *et al*. (2005)**.

Collins (1973) opined that an overwhelming increase in the number of organisms in visceral organs was the cause of death of mice when experimentally inoculated. **Ramdani *et al*. (1990)** observed that on injecting a *P. multocida* type B strain into BALB/c mice, as few as 20 CFU produced an overwhelming septicaemia in mice in less than 30 h, thereby revealing a very rapid *in vivo* multiplication rate. The *P. multocida* A: 1 strain used in this study killed the mice with less than 3 CFU and consequently we could not arrive at a median lethal dose, depicting a very high pathogenicity. Sotoodehnia *et al*. (**Sotoodehnia *et al*., 2004**) also reported similar results with inability to arrive at median lethal dose because of very high virulence of avian *P. multocida* in mice. The post mortem lesions exhibited by dead mice were in conjunction with those reported by **Antony *et al*. (2007**). They also observed that the *Pasteurella* strains isolated from ducks in Kerala which did not even posses any virulence plasmid, killed mice. **Ramanatha (1994)** determined LD_{50} of *P. multocida* A: 1 in mice and obtained a mean value of 14.32 + 0.083 CFU.

The mean lesion score for intestine was similar for all the groups except the seventh group for which it was lower. The lesion score was inconsistent for pericardium and spleen. Heart lesions were more severe in the groups inoculated with more number of organisms. Liver was equally affected in both lower and higher dilutions. The lung lesion score was reduced for groups inoculated with less concentrated inoculums. It was interesting to note that lung lesions induced by our strain were not that severe compared to other organs and this is unusual as *P. multocida* is regarded primarily as a respiratory pathogen. We could draw a correlation between the number of organisms in the inoculum and the lesion severity for organs like heart, intestine and liver.

Colonization and disease causation in a particular host tends to be associated with how well the bacteria get adapted to the host and its ability to overwhelm the host defenses. Resistance to infections is dependent upon a complex equilibrium between many constitutive as well as adaptive defense mechanisms which may be different for each host or even anatomical site of infection and for different pathogens (**Shilo, 1959**). **Hunter and Wobeser (1980)** demonstrated that mallard ducks older than 11 weeks are less vulnerable to *P. multocida* infection than younger ones. Similar age susceptibility was reported in family ducks by **Mbuthia *et al*. (2008)** and opined that more number of clinical signs with increased severity were shown by 4 and 8 weeks old ducklings than the older age groups. This clearly indicates that birds above 4 months have considerable resistance to pasteurellosis and lower doses of bacteria will be cleared by the host immune system. *P. multocida* loses its virulence on storage and multiple sub-culturing. The virulence can be enhanced by an *in vivo* passaging in a living host like mice. In the present study, in vivo passaging was done prior to inoculation to ducklings but not before inoculation to ducks. It may have influenced the outcome of the study, but it is unsure whether that alone contributed to the large variation in virulence in one month and six month old ducks. According to **Matsumoto and Strain (1993)**, *P. multocida* serotypes were able to increase their pathogenicity by bird to bird transmission. In their study, the encapsulated original isolate revealed a mean infectious dose of more than $10^{8.2}$ CFU which after five passages produced 67 per cent mortality with a 10^2 CFU dose. In the present study, only one duck died even in the highest dose of challenge. From this it is obvious that Kuttanad ducks are fairly resistant to pasteurellosis as age advances and they plausibly harbour some disease resistant genes which provide inherent immunity to diseases prevalent in Kuttanad, Kerala. Perusal of available literature did not reveal any genetic studies on disease resistance of Kuttanad ducks or resistance of ducks in general to pasteurellosis and so it warrants extensive studies on their genetic profile and disease resistance to pasteurellosis. The only adult duck that died PI revealed similar gross lesions as that of ducklings but additionally revealed extensive petechiation of mucous membranes of abdominal cavity.

CONCLUSIONS

The median lethal dose of *P. multocida* A:1 isolated from Niranam was 13 CFU in one month old ducklings. Due to inherent disease resistance and extreme lethality of the bacteria, it was not possible to arrive at a medial lethal dose in adult ducks and mice respectively. The dead birds and mice revealed all the classical lesions of pasteurellosis on post mortem examination and organism could be re-isolated from the visceral organs. The animal model should be carefully selected while performing lethal dose studies for it will hugely and single handedly influence the outcome as evidenced in this study. Mouse is not a suitable model for highly virulent strains of *P. multocida* and if used can lead to futile wastage of money, resources and animal lives. The age of the animal host should be optimally selected and correctly judged as it can also act as a critical determinant in obtaining good results.

Acknowledgements: The authors are grateful to the Dean, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India for providing the necessary facilities for conducting this study. The help rendered by Dr. Ambily. R,

Assistant Professor, during the sample collection and procedures is gratefully acknowledged. We also thank the helpers and animal handlers of the Department for the routine management of the animals and birds. The authors acknowledge the Indian Council of Agricultural Research (ICAR), New Delhi, India for providing financial support under the All India Network Programme on Haemorrhagic Septicaemia (AINP-HS) (Project No. R3/66908/2000) and the Dean, College of Veterinary and Animal Sciences, Mannuthy for the intra-mural funding.

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