Int.J.Curr.Microbiol.App.Sci (2019) *8*(12): 680-684

EXCELLENT PUBLISHERS

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 8 Number 12 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.812.089

Determination of Lethal Dose (LD_{50}) of *Riemerella anatipestifer*

Rinsha Balan[1], P. M. Priya[1*], M. Mini[1], Binu K. Mani[2], Vrinda Menon[3] and P. Anitha[4]

[1]Department of Veterinary Microbiology, [3]Department of Veterinary Public Health, [4]Department of Poultry Science, College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur Dt, Kerala-680651, India
[2]Department of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Pookode, Wayanad Dt, Kerala-673576, India

**Corresponding author*

Keywords

Lethal dose, LD50, *Riemerella anatipestifer*, New duck disease, Riemerellosis

Article Info

Accepted:
07 November 2019
Available Online:
10 December 2019

ABSTRACT

New duck disease/ riemerellosis, caused by *Riemerella anatipestifer,* is a predominant disease affecting ducks, causing severe economic loss to the farmers of Kerala. However, the disease has also been reported from other birds. Ducks of all age groups are susceptible, but high mortality was observed in ducklings compared to adults. Vaccination is the only strategy for the control of the disease. As a preliminary approach for the development of vaccine, determination of LD_{50} for *R. anatipestifer* in host system is required and the present study is envisaged for the same. Study groups of birds were inoculated with different concentrations of the bacteria via subcutaneous route. Mortality was recorded two weeks post-inoculation. All the dead birds were examined for specific gross lesions of riemerellosis and re-isolation of the organism was attempted on blood agar from tissues containing lesions. Concentration of 2.5 OD values at 525 nm with a dose of 1 mL per bird subcutaneously was selected as LD_{50}.

Introduction

Riemerellosis, a bacterial disease caused by *Riemerella anatipestifer* is cutting down the profit of duck owners of the state by causing huge mortality among ducklings (Priya *et al.,* 2008). So far, 21 serotypes of the organism were reported globally with little or no cross protection between them. Even after the administration of a course of selected antibiotic, reoccurrence of infection was noticed in some farms resulting in continuous

losses among different batches of ducks. Development of an effective vaccine is the only solution to prevent further economic loss to the duck industry of the state. Field and laboratory trials on inactivated vaccine and laboratory trials on live vaccine have been completed in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Mannuthy. Though they gave good protection, they had inherent drawbacks like painful and cumbersome procedure on inoculating oil adjuvant vaccine and excretion of live organisms on live vaccine administration. To overcome this, use of subunit vaccine is a promising approach.

The outer membrane protein A (OmpA) of *R. anatipestifer* is highly conserved among different serotypes and could be used as a candidate protein for vaccine development. As a pilot study towards vaccine development, the present work is designed for the calculation of LD_{50} to determine the challenge dose in challenge studies.

Materials and Methods

Bacterial isolate

Riemerella anatipestifer isolate (designated as RA1) maintained in the Department of Veterinary Microbiology, CVAS, Mannuthy, as lyophilized form was utilised in the present study.

Revival and subculturing of *R. anatipestifer*

One millilitre of brain heart infusion broth (BHIB) supplemented with bovine serum albumin (BSA) at five per cent concentration was added to the lyophilised vial of RA1 and was streaked onto the blood agar (BA) plates, incubated for 24 to 48 h at 37° C in a candle jar. The colonies obtained were identified based on morphology, cultural and biochemical characteristics (Surya *et al.,* 2016) and it was further confirmed by species-specific PCR assay (Kardos *et al.,* 2006).

Experimental animals

Thirty-two unvaccinated one-day-old ducklings (n=32) (*Anas platyrhynchos*, Kuttanad variety) were procured from a private breeder at Thrissur, Kerala, India. After a week of acclimatisation, the ducklings were randomly assigned to four groups of eight ducklings each.

The first three groups were test groups, while the fourth group served as control. Each group of birds was housed separately in locally made isolator cages.

The control birds were kept in a separate room in order to avoid any chances of cross contamination. The birds were provided with commercial duck feed (formulated at University Poultry Farm, CVAS, Mannuthy) and water ad-libitum.

All the animal experiments were performed with the prior approval of the Institutional Animal Ethics Committee (IAEC) of CVAS, 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.

Determination of LD_{50}

The first three groups were separately inoculated with broth culture of *R. anatipestifer* at a concentration of 2.5, 2.6 and 2.7 OD values at 525 nm with a dose of one millilitre per bird subcutaneously (Table 1).

The fourth group served as control. Mortality was recorded up to one week post-inoculation. All the dead ducklings were examined for specific gross lesions of

riemerellosis and re-isolation of the organism on BA from heart blood and liver was attempted.

Results and Discussion

Identification of the isolates

Based on cultural, morphological, biochemical and molecular characteristics, the isolate was identified as *R. anatipestifer*. On yielding an amplicon of 546 bp, it was further confirmed by species- specific PCR assay.

Lethal dose in ducklings

Broth culture of RA1 with a concentration of 2.5 OD values at 525 nm, administrated subcutaneously at the rate of one millilitre in to one-week-old ducklings was selected as LD_{50}. Out of the eight birds inoculated, exactly half of the birds died within a week. Thus, the LD_{50} could be obtained directly from the observation. The details of dead and live duckling of each group were furnished in Table 2.

The gross lesions observed in experimentally infected ducklings were fibrinous pericarditis, perihepatitis and air-sacculitis with severely congested liver and spleen (Fig. 1). Microscopic examination revealed bipolar organisms from blood smear and colonies suggestive of *R. anatipestifer* from internal organs of all the succumbed ducklings could be observed on BA. The control birds did not reveal any bacterial growth following culturing the collected tissues from them.

Although, ducks immunised against *R. anatipestifer* infection with inactivated bacterins and live or cell free filtrate bacterins provide protection against homologous serotype, they failed to provide protection against heterologous serotype (Layton and Sandhu, 1984; Pathanasophon *et al.,* 1996). To overcome this limitation, use of subunit vaccine could be a promising approach. Literature reveals that the outer membrane protein A (OmpA) of *R. anatipestifer* is highly conserved among different serotypes and could be used as a candidate protein for vaccine development and prior to development of the vaccine, determination of LD_{50} is necessary.

In the present study, LD_{50} was calculated for the RA1 strain to determine the challenge dose in challenge studies. Broth culture of RA1 having a concentration of 2.5 OD values at 525 nm administrated at the rate of one millilitre per bird subcutaneously was selected as the LD_{50}. Out of the eight inoculated birds, exactly half of the birds died within a week. Thus, the LD_{50} could be obtained directly from the observations. Jayakumar (1998) and Sheethal (2012) also used similar route of inoculation (subcutaneous) for the determination of LD_{50} of *P. Multocida* A: 1 isolate in one- month-old ducklings. Mortality was recorded up to one week post-inoculation and out of the six inoculated birds of the dilution 10^{-7}, exactly half of the birds died within one week post-inoculation and they also calculated the LD_{50} directly from the observations.

Table.1 Determination of LD_{50}

Groups	OD_{525}	Age, dose and route
1	OD_{525}= 2.4	Ten-day-old, 1 mL, subcutaneous
2	OD_{525}= 2.5	Ten-day-old, 1 mL, subcutaneous
3	OD_{525}= 2.6	Ten-day-old, 1 mL, subcutaneous
4	Control	Uninoculated

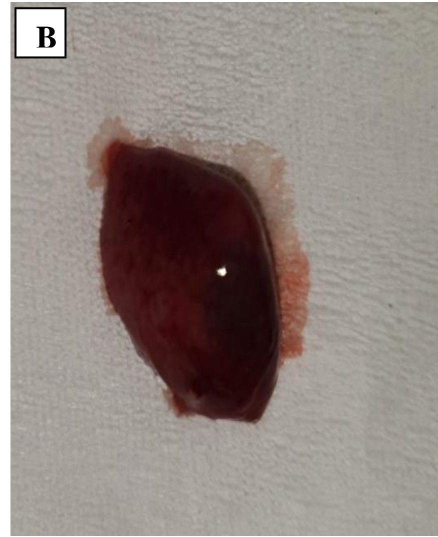
Table.2 Group- wise details of dead and alive ducklings

Groups	OD_{525}	No. of birds tested	No. of died	No. of live
1	OD_{525}= 2.4	8	3	5
2	OD_{525}= 2.5	8	4	4
3	OD_{525}= 2.6	8	5	3
4	Control	8	nil	8

Fig.1 Gross lesions on experimental inoculation with *R. anatipestifer*

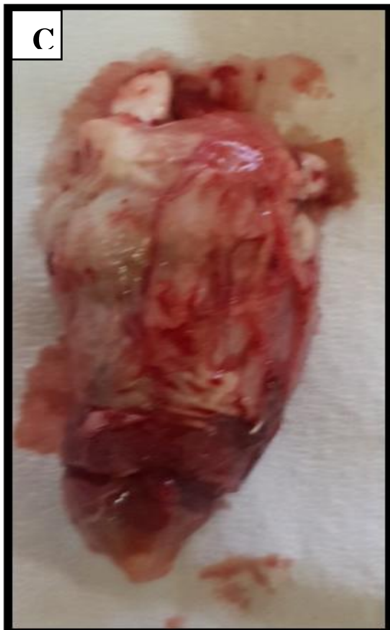
(A) air-sacculitis with severely congested liver

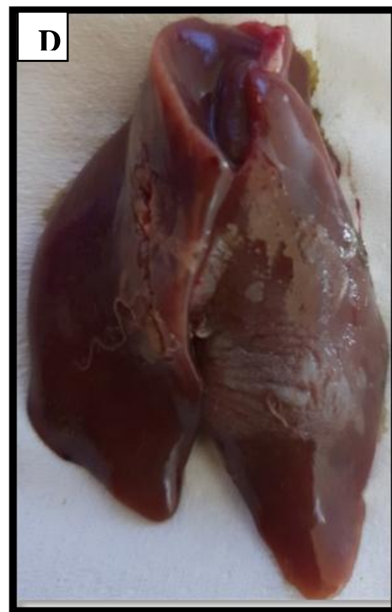
(B) congested spleen



(C) fibrinous pericarditis

(D) perihepatitis





The lesions observed in experimentally infected ducklings were typical of riemerellosis *i.e.,* fibrinous pericarditis, perihepatitis and air-sacculitis with severely congested liver and spleen. Blood smears and impression smears from organs revealed bipolar stained organisms. Similar lesions were observed by Pickrell (1966), Liu *et al.,* (2013) and Chu *et al.,* (2015) in ducks infected with *R. anatipestifer*.

The lethal dose of *R.anatipestifer* was selected as concentration of 2.5 OD values at 525 nm in eight day old ducklings. The dead birds revealed all the classical lesions of riemerellosis on post mortem examination and organism could be re-isolated from the organs.

References

Chu, C., Liu, C., Liou, J., Lee, J. and Cheng, L. 2015. Development of a subunit vaccine containing recombinant *Riemerella anatipestifer* outer membrane protein A and CpG ODN adjuvant. *Vaccine.* 33: 92-99.

Jayakumar, P. S. 1998. Comparative efficacy of different vaccines against pasteurellosis in ducks. *M.V.Sc. thesis*, Kerala Agricultural University, Thrissur, 126p.

Kardos, G., Nagy, J., Antal, M., Bistyak, A., Tenk, M. and Kiss, I. 2006. Development of a novel PCR assay specific for *Riemerella anatipestifer. Lett. Appl. Microbiol.* 44: 145-148.

Layton, H.W. and Sandhu, T. S. 1984. Protection of ducklings with a broth-grown *Pasteurella anatipestifer* bacterin. *Avian Dis*. 28: 718-726.

Liu, H., Wang, X., Ding, C., Han, X., Cheng, A., Wang S. and Yu, S. 2013. Development and evaluation of a trivalent *Riemerella anatipestifer*-inactivated vaccine. *Clin. Vaccine Immunol.* 20(5): 691.

Pathanasophon, P., Sawada, T., Pramoolsinsap, T. and Tantcharoenyos, T. 1996. Immunogenecity of *Riemerella anatipestifer* broth culture bacterin and cell-free culture filtrate in ducks. *Avian Pathol.* 25: 705-719.

Pickrell, J.A. 1966. Pathologic changes associated with experimental *Pasteurella anatipestifer* infection in ducklings. *Avian Dis.* 10: 281-288.

Priya, P.M., Pillai, D.S., Balusamy, C., Rameshkumar, P. and Senthamilselvan, P. 2008. Studies on outbreak of new duck disease in Kerala, India. *Int. J. Poult. Sci.* 7: 189-190.

Sheethal, G.M. 2012. Comparative efficacy of oil adjuvant vaccines against duck pasteurellosis. *M.V.Sc thesis*, Kerala Veterinary and Animal Sciences University, Pookode, 80p.

Surya, P.S., Priya, P.M. and Mini, M. 2016. Biotyping and antibiogram of *Riemerella anatipestifer* from ducks in Kerala. *Biosci. Biotech. Res. Comm.* 9(3): 457-462.

How to cite this article:

Rinsha Balan, P. M. Priya, M. Mini, Binu K. Mani, Vrinda Menon and Anitha, P. 2019. Determination of Lethal Dose (LD_{50}) of *Riemerella anatipestifer*. *Int.J.Curr.Microbiol.App.Sci.* 8(12): 680-684. doi: https://doi.org/10.20546/ijcmas.2019.812.089