



Therapeutic Trials of Johne's Disease With Amikacin and Cinnamon in Experimentally Challenged Rabbits

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ABSTRACT

Johne's disease has its economic importance worldwide because of heavy treatment cost and poor outcomes. This study is conducted on experimentally challenged rabbits to observe the efficacy of amikacin and cinnamon. A total of 40 SPF (specific pathogen free) rabbits were selected at the age of two months. Rabbits were given experimental challenge with oral inoculum of pure culture of MAP. These rabbits were divided into four groups; three test and one control group. Histopathological lesions were observed before and after the therapeutic trials and lesions scoring was given according to the standard. On the basis of the changes in histopathological lesions, the effect of these therapeutic agents was noted. Amikacin showed marked differences in terms of decrease in severity of lesions whether used alone or in combination with cinnamon in rabbits. It was observed that amikacin and cinnamon having synergistic effect in rabbits and are economically important drugs in treatment of Johne's disease.

Article Information

Received 25 October 2018

Revised 01 March 2019

Accepted 27 March 2019

Available online 03 December 2019

Authors' Contribution

MA conducted the research.

AZD supervised the study.

MI and AAA were member of supervisory committee. BM helped in histopathology. MH helped in laboratory work. MU helped in write-up of the manuscript

Key words

Treatment, Johne's disease, Paratuberculosis, Histopathology, Rabbits

INTRODUCTION

Mycobacterium avium subsp. Paratuberculosis (MAP) causes severe diarrheic disease in ruminants and is potentially hazardous for human health (Naser *et al.*, 2004). Passive fecal shedding of MAP has been noted in ruminants irrespective of their infection status and animals exposed to MAP show positive fecal sample test even when never become infected (Dennis *et al.*, 2011). Keeping this in mind histopathological confirmation of disease is better option than others. The identification through biopsies may be helpful in human health (Chiodini *et al.*, 1996) because of potential link of MAP with human Crohn disease (Weddell *et al.*, 2016). Culture of organism is gold standard test for diagnosis but PCR is more reliable and better than culture and identification through some assays (Alonso-Hearn *et al.*, 2009). MAP is naturally resistant to most of the antibiotics. Poorly supported information is available regarding the evidence of successful treatment because very few experiments have been performed (Van Ingen *et al.*, 2012). Currently there is no drug recommended for the treatment of Johne's disease though

in a previous study it was reported that infection with the probiotic bacterium Dietzia, alone or in combination with steroid, i.e., dexamethasone, inhibited the growth of the *Mycobacterium* in cattle (Click *et al.*, 2011). The heat-treated colostrum was also found positive for reducing the infection of *Map* in dairy cow (Verheghe *et al.*, 2017). In advanced and previously treated complex cases, aminoglycosides were preferred (Kobashi *et al.*, 2007). Amikacin in combination gave good results in treatment of disease (Griffith *et al.*, 2007). Cinnamon oil also has good antibacterial activity and it inhibits the growth of MAP (Wong *et al.*, 2008). Significant effect of cinnamon has been found against MAP and its active ingredient cinnamonaldehyde decreased the intracellular ATPs and increased phosphate leakage (Nowotarska *et al.*, 2017). Histopathological lesion studies of tissues from infected animals can be considered as the gold standard method for the diagnosis of Johne's disease and should be used to confirm the disease instead of bacteriology (Hope *et al.*, 2000).

MATERIALS AND METHODS

Study animals

Rabbits were used as study model for therapeutic trials of Johne's disease as the same strain of MAP causes the disease in rabbits and dairy cattle. Forty SPF (specific

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0030-9923/2020/0001-0001 \$ 9.00/0
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pathogen free) rabbits were purchased from Animal House, Veterinary Research Institute. For the study, permission was obtained from Ethical Committee of Advance Studies, University of Veterinary And Animal Sciences, Lahore. All rabbits two month of age were kept separately in WTO quality assurance building. All rabbits were provided with similar feed and water. These rabbits were divided into four groups each group containing ten rabbits.

Specific MAP antigen was taken from Microbiology Department of University of Veterinary and Animals sciences and cultured on Herald egg yolk media with mycobactin J to introduce the disease in rabbits. Each rabbit was orally challenged with 0.4×10^6 for consecutive ten days with a total dose of 4 ml. Signs of diarrhoea were observed for few months. Post mortem-I was performed in rabbits with clinical signs of disease before therapeutic trials. Then different drugs were given to each group for four weeks and after treatment the decrease in diarrheic episode was seen in different groups. Postmortem-II was performed in rabbits from each group after therapeutic trial and findings were noted.

Histopathological study of tissues

Rabbits with clinical signs of disease were selected from each group for post mortem to find out the histopathological lesions. The tissues were cut finely with the help of microtome and 5 μ m tissue sections were studied embedded in paraffin and stained with eosin-hematoxylin. Lesions were given the scores 0-4 in case of gross lesions and 0-3 in case of intestinal lesions by pathologist following the criteria already established (Perez *et al.*, 1996). The grade 3 score were further classified into 3(a) paucibacillary 3(b) multibacillary and 3(c) severe bacillary. The lesions in the lymph nodes were also graded into 1. mild focal, 2. mild multifocal and 3. severe multifocal.

Statistical analysis

Data obtained was analysed by applying ANOVA test to check the efficacy of drugs by SPSS and Figures were made by using the software graphpad prism.

RESULTS

Histopathological findings were studied in experimentally challenged rabbits that were inoculated with 0.4×10^6 CFU for consecutive ten days. Signs of the disease started appearing after three months of inoculation. A total of 19 (47.5%) out of 40 rabbits were infected with signs of diarrhoea. Consistency of diarrhoea found varies from semisolid to pasty or liquid. Episodes of diarrhoea was different in numbers individually in each group varying from 2 to 6. After inoculation the post mortem

lesions were observed in diarrheic rabbits in different part of GIT i.e. ileum, cecum, ileocecal valve and lymph node. Once the signs of disease had appeared the treatment trials were started. In each group a specific drug was given up to 6 weeks. Group A was given Amikacin @10mg/kg body weight in the form of intramuscular injection at quadriceps femoris muscle of rabbit. In group B the combination of Amikacin I/M and Cinnamon @ 250mg/kg PO was given. In group C only cinnamon @ 250mg/kg was given to treat the rabbits and group D was kept as control and no drug was given to this group.

Post mortem of rabbits was performed at two levels of the study i.e. Post mortem-I was performed after appearance of sign of diarrhoea and post mortem-II after the completion of therapeutic trials. In post mortem-I three rabbits from each group were slaughtered and post mortem lesions were noted. For that purpose scores were given to each type of lesion. Following is given the detail of lesion scoring.

In each group three diarrheic rabbits were slaughtered and postmortem lesions were observed in each case. Group A showed 3 rabbits with mucosal corrugation marked in nature. Three rabbits with prominent lymphatics marked in nature. One rabbit with lymph adenopathy mild in nature and one rabbit emaciated. In group B three rabbits containing lesion corrugation moderate in nature, 3 rabbits with prominent lymphatics, moderate in nature and no rabbit with lesion three. In group C, 2 rabbits with corrugation, mild and two rabbits with prominent lymphatics mild in severity and one rabbit with lymph adenopathy. In case of control group no type of lesion was observed. Gross lesion scoring in diarrheic rabbits before trials was given in Table I.

Table I. Gross lesion scoring in diarrheic rabbits (Before trials).

Groups	diarrheic Total	PM-I performed	Lesion scoring				
			0	1	2	3	4
A	7	3 out of 7 diarrhea positive	0	3(3+)	3(3+)	1(1+)	1(2+)
B	6	3 out of 6	0	3(2+)	3(2+)	-	1(1+)
C	6	3 out of 6	0	2(1+)	2(1+)	1(1+)	
D	0	3 non-diarrheic	10	-	-	-	-

PM-I, rabbits slaughtered before treatment

Post mortem-II was performed after the therapeutic trials; three rabbits were slaughtered from each group and

lesions were observed in each case. It was found from the observations that there were some improvements in lesions in post mortem-II. In Group A, 3 rabbits were seen with corrugation moderate in nature. Two rabbits with prominent lymphatics moderate in nature. No rabbit with lymph adenopathy and emaciated. In group B three rabbits containing mild in nature, 2 rabbit with prominent lymphatics mild in nature and one rabbit with lymph adenopathy and no emaciated. In group C, 2 rabbits with lesion one, mild and two rabbits with lesion two mild in severity and one rabbit with lymph adenopathy mild. In case of control group no type of lesion was observed. Gross lesion scoring in diarrheic rabbits after trials are described in Table II.

Table II. Gross lesion scoring in diarrheic rabbits after trials.

Groups	Diarrheic	Total	PM-11	0	1	2	3	4
A	4	3 out of 4	0	3(2+)	3(2+)	1(1+)	1(3+)	
B	3	3 out of 3	0	3(1+)	2(2+)	-	-	
C	3	3 out of 3	0	2(1+)	2(1+)	1(1+)	1(1+)	
D	0	3 out of 7	10	-	-	-	-	

PM-1, rabbits slaughtered before treatment

Figure 1A represents that amikacin having good efficacy among all drugs used in combination or alone in case of self-induced MAP infection to rabbit model. Efficacy of amikacin alone ranked the highest among all drugs (P value > 0.05 suggested that no significant results in case of gross lesion.)

Histopathological lesion scoring was observed in intestine in diarrheic rabbits before trials. So, post mortem-I was performed before the therapeutic trials; three rabbits were slaughtered from each group and lesions were observed from each case. It was found from the observations that in group A, three rabbits with macrophages of moderate in nature. Two rabbits found with lesion in peyer patches and moderate in severity. One rabbit was noticed with lesion in peyer patches having bigger granuloma involved more villi which were moderate in nature. One rabbit with granulomatous enteritis, moderate in nature and 2 rabbit were with granulomatous enteritis but with predominant lymphocyte severe in nature. In group B, 3 rabbits having macrophages, moderate in nature and 3 rabbits with lesion in peyer patches, moderate in nature and 3 rabbit with granuloma mild in nature and one rabbit with enteritis. In group C, 1 rabbit having macrophages; moderate and one

rabbit with lesion in peyer patches moderate in severity; 3 rabbit with granuloma moderate; no rabbit with enteritis plus granuloma and one rabbit with granuloma, enteritis and lymphocyte mild in severity. In case of control group, no lesion was observed. Histopathological lesion scoring of intestines in diarrheic rabbits before trials are described in Table III.

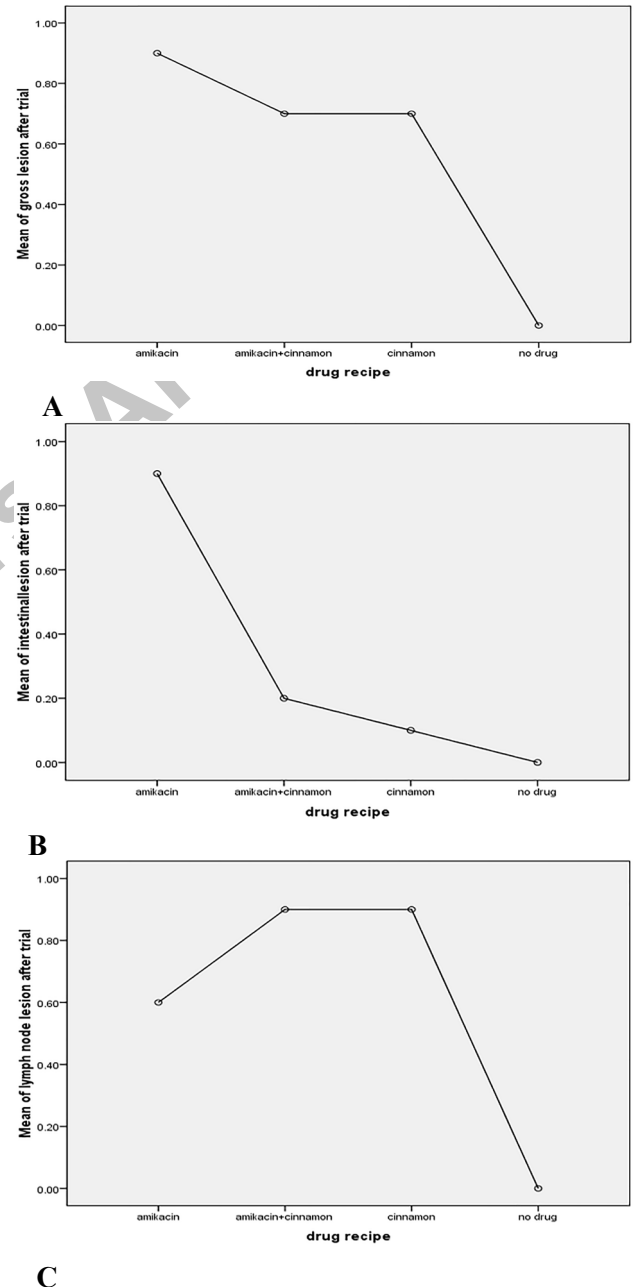


Fig. 1. Efficacy of amikacin (A) alone ranked the highest among all drugs and on the top in case of intestinal lesions (B) and case of lymph node lesions (C).

Table III. Histopathological lesion scoring of intestine in diarrheic rabbits (before trials).

Groups	Total diarrheic	PM-I performed	Lesion scoring					
			0	1	2	3(a)	3(b)	3(c)
A	7	3/7	0	3 (2+)	2(2+)	2(2+)	1(2+)	2(3+)
B	6	3/6	0	3(2+)	3(2+)	3(1+)	1(1+)	-
C	6	3/6	0	1(2+)	1(1+)	3(2+)	-	1(1+)
D	0	3/10	10	-	-	-	-	-

Lesion scoring: 0, No lesion; 1, granuloma +macrophages; 2, lesion in peyer patches+granuloma in lamina propria; 3(a), lesion in peyer patches+ bigger granuloma involved more villi; 3(b), granulomatous enteritis, epithelioid cells giving mosaic like appearance of mucosa; 3(c), granulomatous enteritis but with predominant lymphocyte. Severity of findings: (Mild +1, Moderate +2, Marked +3)

Table IV. Histopathological lesion scoring of intestine in diarrheic rabbits (after trials).

Groups	Total Diarrheic	PM-11	Lesion scoring					
			0	1	2	3	4	5
A	4	3/4	0	3(1+)	1(2+)	2(1+)	1(2+)	2(3+)
B	3	3/3	0	1(2+)	2(1+)	1(1+)	-	-
C	3	3/3	0	1(1+)	-	2(2+)	-	1(1+)
D	0	3/7	10	-	-	-	-	-

Histopathological lesion scoring was observed in intestine in diarrheic rabbits after trials. So, post mortem-II was performed after the therapeutic trials; three rabbits were slaughtered from each group and lesions were observed in each case. It was found that in group A, three rabbits with macrophages of mild in nature; one rabbit found with lesion in peyer patches and moderate in severity; two rabbits with lesion in peyer patches having bigger granuloma involved more villi moderate in nature; one rabbit with granulomatous enteritis, moderate in nature; and two rabbits were with granulomatous enteritis but with predominant lymphocyte severe in nature. In group B, three rabbits having macrophages moderate in nature; three rabbits with lesion in peyer patches, moderate in nature; three rabbits with granuloma mild in nature; one rabbit with enteritis. In group C, one rabbit having macrophages, moderate; one rabbit with lesion in peyer patches moderate in severity; three rabbits with granuloma moderate; no rabbit with enteritis plus granuloma; and one rabbit with granuloma, enteritis and lymphocyte marked in severity. In case of control group, no lesion was observed.

Histopathological lesion scoring of intestines in diarrheic rabbits after trials are given in [Table IV](#).

In group B, it was found that there was significant difference of histopathological lesions in intestine by the use of the drug though the results were non-significant statistically. The graph represents that amikacin has good efficacy among all the drugs used to cure self-induced MAP infection to rabbit model when intestinal lesions were taken as parameter ([Fig. 1B](#)).

Lesion were also noted in mesenteric lymph node in diarrheic rabbits before trials through histopathology. Post mortem-I was performed before the therapeutic trials; three rabbits were slaughtered from each group and lesions were observed in each case. It was found from the observations that in group A, two rabbits with mild focal lesion; three rabbits found with mild multifocal lesion; one rabbit with severe multifocal to diffuse granuloma. In group B, three rabbits had mild focal lesion; three rabbits with mild multifocal lesion; and two rabbits with severe multifocal to diffuse granuloma. In group C, three rabbits with score mild focal; two rabbits with mild multifocal; and three rabbits with severe multifocal. In case of control group, no lesion was found. Histopathological lesions scoring of mesenteric lymph node in diarrheic rabbits before trials are given in [Table V](#).

Table V. Histopathological lesions scoring of mesenteric lymph node in diarrheic rabbits (before trials).

Groups	Total diarrheic	PM-I performed on	Lesion scoring			
			0	1	2	3
A	7	3/7	0	2	3	1
B	6	3/6	0	3	3	2
C	6	3/6	0	3	2	3
D	0	3/10	10	-	-	-

Scoring of focal lesions: 0, No lesion; 1, mild focal lesion; 2, mild multifocal lesion; 3, severe multifocal to diffuse granuloma. Severity of findings: (Mild +1, Moderate +2, Marked +3)

Table VI. Histopathological lesions scoring of mesenteric lymph node in diarrheic rabbits (after trials).

Groups	Total Diarrheic	PM-11	Lesion scoring			
			0	1	2	3
A	4	3 out of 4	0	1	3	1
B	3	3 out of 3	0	1	1	1
C	3	3 out of 3	0	2	2	3
D	0	3 out of 7	10	-	-	-

Histopathological lesions were also observed in mesenteric lymph node in diarrheic rabbits after trials. Post mortem-II was performed after the therapeutic trials; three rabbits were slaughtered from each group and lesions were observed in each case. It was found from the observations that in group A, one rabbit was with mild focal lesion; three rabbits with mild multifocal lesion; one rabbit with severe multifocal to diffuse granuloma. In group B, one rabbit containing mild focal lesion; one rabbit with mild multifocal lesion; one rabbit found with severe multifocal to diffuse granuloma. In group C, two rabbits with score mild focal; two rabbits with mild multifocal; and three rabbits with severe multifocal. In case of control group, no lesion was observed. Histopathological lesions scoring of mesenteric lymph node in diarrheic rabbits after trials are given in Table VI. The efficacy of amikacin and cinnamon was on the top in case in lymph node lesions is also shown in Figure 1C. The histo-pathological changes are shown in Figure 2.

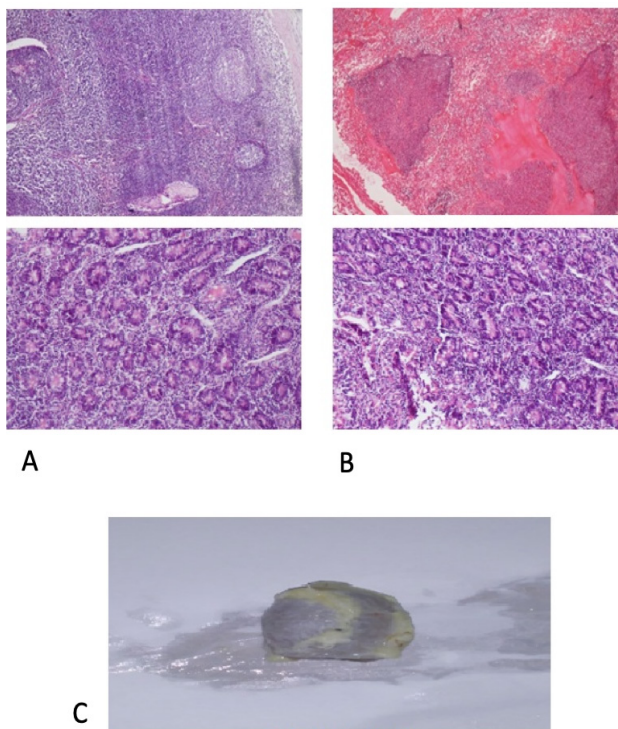


Fig. 2. Infiltration of Lymphocytes in the intestine. (A) macrophages, (B) Granuloma and (C) Enlarges lymph node.

DISCUSSION

Rabbits have been used as an excellent model to conduct therapeutic trials in different previous studies.

Rabbits for therapeutic trials have been used at an early age of life to incorporate the disease. In previous studies, animals were infected on their first day of life. Experimental challenge of Johne's disease with single dose of inoculum works but multiple dosing schemes have been reported in some recent studies. In this study, the number of MAP were used @ of 4×10^6 CFU. In general, the number of MAP would range from 5×10^6 (Juste *et al.*, 1994) to 3×10^7 CFU (Hines *et al.*, 2007) per mg pelleted wet weight and averaging approximately 1×10^7 CFU/mg (Sweeney and Hines, unpublished information). A retrospective estimate of the number of CFU inoculated should be made by performing colony counts plated on serial dilutions (Hines *et al.*, 2007).

Histopathological lesion studies of infected animals can be considered as the gold standard method for the diagnosis of Johne's disease and should be used to confirm the disease instead of bacteriology (Hope *et al.*, 2000). Histopathological changes occur in mucosa and sub mucosa of intestine with the infiltration of lymphocyte and macrophages and formation of granuloma in gastrointestinal tract and adjacent lymph nodes. Inflammation, corrugation, granulomatous material and enlarged lymph nodes in these areas were frequently seen in positive rabbits.

Previously, very few studies were conducted on therapeutic trials on live animal models. Although some in vitro trials showed good results of different antibiotics and these drugs were successfully used to show the efficacy of drugs against MAP in vitro trials. In these in vitro studies, azithromycin showed maximum results, i.e., 100%, amikacin 83%, rifampicin 44.4%, and Rifabutin 38.8%. But, in general, Azithromycin is not a cost-effective drug. On the other hand Amikacin is a cost effective drug and MAP is susceptible to amikacin and showed better results than others antibiotics in vitro studies (Krishnan *et al.*, 2009). Cinnamonaldehyde the active ingredient of cinnamon has good antimicrobial activity. It goes inside the cell and decreases adenosine triphosphate ATPs. While in this study the test statistics suggested the decrease in lesions which is a positive approach in curing paratuberculosis in rabbits (Nowotarska *et al.*, 2017).

Degree of appearance of signs in rabbits after experimental challenge varies in various studies. In present study, signs appeared after three months of inoculation. In another study, four adult and sixteen juveniles were orally given the challenge with 1 ml of 10^8 CFU and signs were appeared after four months of inoculation. Again, in another trial, thirteen rabbits were kept for the purpose and orally inoculum 3.6×10^8 was given for two days and signs were appeared after three months in nine rabbits.

Histopathological findings were also studied in

experimentally challenged rabbits. After three months of inoculation, the post mortem lesions were observed in diarrheic rabbits in different part of gastrointestinal tract, i.e., ileum, cecum, ileocecal valve and lymph nodes. The chronic diarrheic lesions are very much similar to those which are commonly found in cattle (Buergelt *et al.*, 1978).

These lesions include granulomas, focal lesions multifocal lesions, giant cells, macrophages and fibrous tissues and similar type of multifocal lesions found in ruminants (Chidioni *et al.*, 2010). These similar kinds of findings of enteritis in rabbits and cattle suggest that rabbits are good model for study of lesions in ruminants and other animals (Mokresh *et al.*, 1989).

These lesions were categorized into different categories i.e. mild, moderate and marked in different parts of gastrointestinal tract and adjacent lymph nodes. After completion of therapeutic trials with amikacin and cinnamon again findings of these lesions were recorded and compared with the previous one in all groups. There was considerable difference in lesions before and after trials. This decrease in lesions may be suggested as a positive sign towards the treatment of Johne's disease.

ACKNOWLEDGEMENT

Authors acknowledge the Department of Microbiology, University of Veterinary and Animal Sciences Lahore for providing pure culture of MAP and Pathology section of WTO Quality Assurance Laboratory to help performing the histopathology and also providing cages to keep the rabbits separately.

CONCLUSION

The results of this study showed that severity of lesions markedly decreased with the administration of amikacin and cinnamon in experimentally challenged Johne's diseased rabbits. Their use may also be helpful in other affected ruminants, i.e., sheep, goats and especially bovines.

Statement of conflict of interest

The authors declare no conflict of interest.

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