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Efficacy of Antibiotics (Doxycycline and Kanamycin) against Japanese encephalitis virus infection

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Abstract. The study evaluated antiviral efficacy of antibiotics- Doxycycline and Kanamycin against Japanese encephalitis virus (JEV) infection *in vivo*. Adult Swiss albino mice (4- 6 weeks) were used. Mice were distributed in four groups- control group (A), only drug group (B), JEV infected group (C) and JEV + drug treated group (D). Mice were given intravenous inoculation of JEV strain P20778. Doxycycline was given via intra peritoneal (i. p.) route at 50 mg/kg dose. Kanamycin was given to mice via subcutaneous (s. c.) route at 20 mg/kg dose. All drug dosages were administered at 24 hr, 48 hr and 96 hr post infection (p.i.) twice a day (BID) for upto 14 days. The mice were monitored for 21 days. The viral load was determined by plaque assay. Viral RNA load and cytokine levels were determined. The infected mice died by 8 days of infection. Doxycycline treatment at 50 mg/kg dose after 24 hr p.i. lowered disease progression, prolonging the survival of the animals by a week. Antiviral effect was evident with reduction of progeny plaque formation. The plaque formation was reduced at 24 hr p.i. compared to virus group. Doxycycline inhibited viral RNA replication. Doxycycline was able to moderately modulate proinflammatory cytokines. Kanamycin administration was less effective. Thus, the studies demonstrated encouraging results in treatment of JEV infection by Doxycycline. It indicated that Doxycycline delayed the disease progression. Thus, the findings suggest that Doxycycline could be used as an adjuvant treatment against JE.

INTRODUCTION

Japanese encephalitis (JE) is a disease of major public health importance. Approximately 3 billion people (60.0% of world population) reside in JE endemic areas (Theodore, 1996). Annually, 35,000 to 50,000 cases are reported with up to 15,000 deaths (Schweitzer *et al.*, 2009). JE disease is caused by Japanese encephalitis virus (JEV), a member of JEV serocomplex of genus *Flavivirus*, family *Flaviviridae* (Solomon *et al.*, 2003). It is a small, enveloped virus with a single-stranded, positive-sense RNA genome of approximately ~11kb (Lindenbach *et al.*, 2001). JEV is transmitted in an enzootic cycle comprising mosquitoes, ardeid water birds (e.g. herons and pond egrets) which serve as reservoir hosts and pigs are considered as the main amplifying hosts. The principal mosquito vector species belong to *Culex* species. Humans are the incidental dead end host (Changbunjong *et al.*, 2013).

Recently, certain antibiotic compounds, mainly certain tetracyclines and aminoglycosides compounds have been successfully proved beneficial against viral infection namely Dengue virus (DENV), West Nile virus (WNV) and Reovirus (Yang *et al.*, 2007; Michaelis *et al.*, 2007;Burns *et al.*, 2005). In a previous report, a case study of JEV infection in Swiss albino mice showed treatment with Minocycline, a tetracycline compound, provided protection against the infection. Minocycline prevented sequelae of infection, including restricted movement, body stiffening, piloerection, hind limb paralysis and tremor (Mishra *et al.*, 2008). We report the activity of Doxycycline; a tetracycline and Kanamycin; an aminoglycoside against JEV infection *in vivo*.

MATERIAL AND METHODS

2.1. Drugs (Doxycycline and Kanamycin) administration in an experimental mice model of JEV

Adult Swiss albino mice (4-6 weeks) of either sex were used. Mice were distributed in four groups- control group (A), only drug group (B), JEV infected group (C) and JEV + drug treated group (D). A total of 15 mice in each group were included. Group A received PBS. Mice were infected with 3 x 10^5 pfu of JEV strain intravenously (via tail vein). The dose concentration for Doxycycline was 50 mg/kg (Bastos *et al*., 2007). Doxycycline was given via intra peritoneal (i. p.) route. Kanamycin was administered at 20 mg/kg dose (Nishi *et al*., 1980). Kanamycin was given to mice via subcutaneous (s. c.) route. All drug dosages were administered at 24 hr, 48 hr and 96 hr post infection (p.i.) twice a day (BID) for upto 14 days. The mice were monitored for 21 days. All experiments were performed according to protocol approved by the Institutional Animal Ethics Committee (IAEC) vide letter no: RMRC/DIB/IAEC (Animal)/2013-14/126 date. 16.04.2013.

2.2. Virus titration / Plaque assay

The viral load in CNS of infected mice was determined. Brains were dissected, cooled on ice, homogenized and titrated for virus by plaque formation on BHK 21 cell monolayers. Monolayers were inoculated with 10 fold dilutions of virus sample made in MEM containing 2% FCS and incubated for 1 hr at 37ºC with occasional shaking. The inoculum was removed by aspiration and the monolayers were overlaid with MEM containing 2% FCS and 0.9% CMC. Plates were incubated at 37ºC for 3 days. The cells were fixed with 10% formaldehyde, stained with amido black and the plaques were counted.

2.3. Quantitative estimation of viral load by Quantitative PCR (qPCR)

JEV E III region specific primers used were F 5'-GGGAGTGATGGCCCCTGCAAAATT-3' and R 5'-TCCAATGGAGCCAAAGTCCCA GGC-3' respectively (Huang *et al*., 2008). β-actin gene primers for mouse are: 5'-TCCTGTGGCATCCACGAAACT-3' and 5'-GAAGCATTTGCGGTGGACGAT-3' (Srichai *et al*., 2008). Viral RNA load in antibiotics treated cells was compared to untreated controls and was normalized to the reference gene (β-actin). The $2^{-\Delta\Delta CT}$ method was used to analyze relative changes in JEV RNA levels from real-time quantitative RT-PCR experiments (Schmittgen *et al*., 2000; Winter *et al*., 1999; Livak *et al*., 2001; Yilmaz *et al*., 2012).

2.4. Cytokine bead evaluation assay

The BD mouse cytokine bead array kit (Mouse Th1/Th2/Th17 Cytokine Kit) was used to quantitatively measure cytokine levels from mouse brain lysates. The assay was performed according to manufacturer's instructions and analyzed on the FACS Canto II (BD Biosciences, USA).

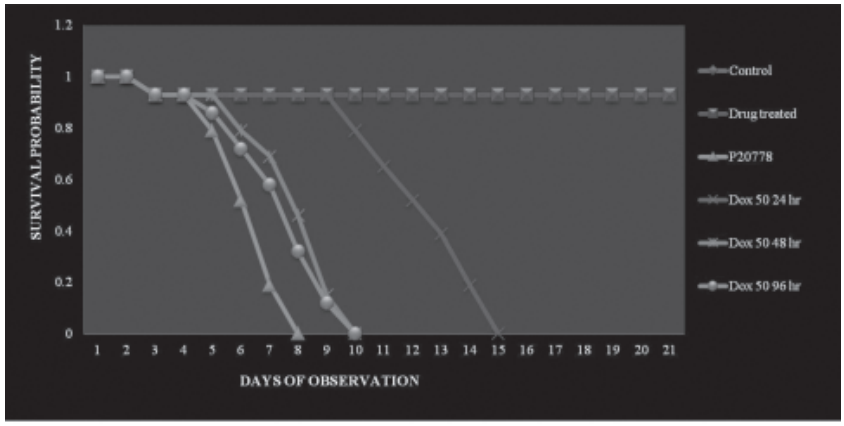
2.5. Statistical analysis

Statistical analysis was done with the help of SPSS v.16 software. An unpaired student's t test was used for comparisons between 2 groups. One way ANOVA test was used to determine significance among the groups. Value of $p<0.05$ was considered significant. Mice survival analysis was done by Kaplan Meier method.

RESULTS

3.1. Drug's efficacy against JEV in mice model

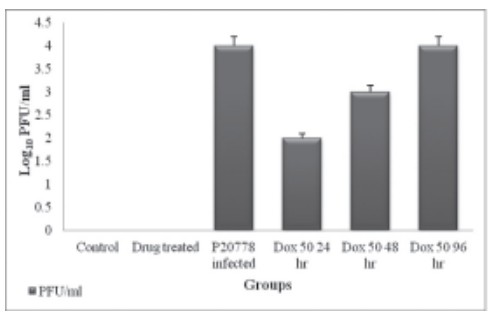
The experimental mice were monitored for upto 21 days. Both the control group and drug treated group animal were in proper health condition and survived for the entire 21 days. JEV infected animal group died by 8 days of infection. Mice treated by Doxycycline 24 hrs p.i. survived upto 15 days. Thus, it was found that the drug Doxycycline substantially delayed the progression of the disease ($p<0.05$). JEV infected mice treated with Kanamycin survived for one more day compared to the non-drug treated group (Fig. 1).



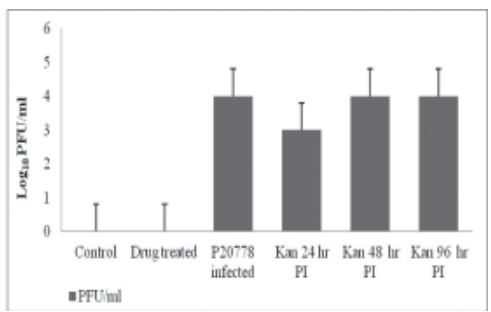
(a) Kaplan Meier Survival Curve of experimental animal groups treated with Doxycycline.

(b) Kaplan Meier Survival Curve of experimental animal groups treated with Kanamycin.

Figure 1. Kaplan Meier survival curve of animal groups treated with (a) Doxycycline and (b) Kanamycin.



(a) Plaque titration (PFU/ml) of experimental groups treated with Doxycycline.



(b) Plaque titration (PFU/ml) of experimental groups treated with Kanamycin.

Figure 2. Plaque titration (PFU/ml) of experimental groups treated with (a) Doxycycline and (b) Kanamycin.

3.2. Effects of Drugs on JEV viral titer

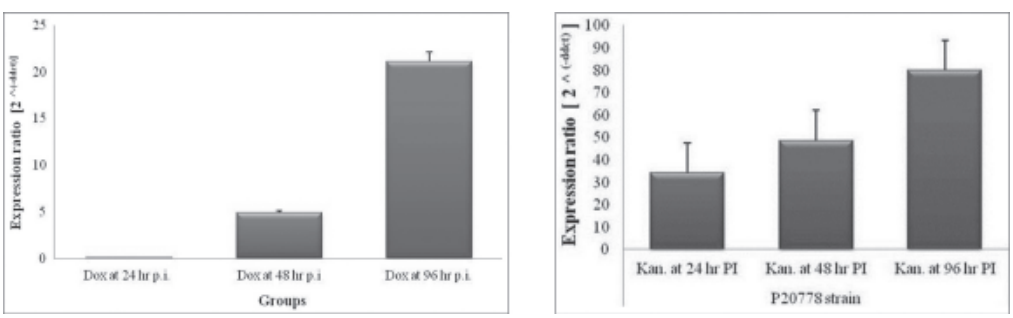
JEV viral titer of infected mice group was found to be 2x 10^4 pfu /ml. Treatment with Doxycycline reduced the virus titers to 1 x 10^2 pfu/ml at 24 hr p.i. ($p<0.05$). Treatment with Kanamycin also reduced the virus titers in treated mice model to 1 x 10^3 pfu/ml at 24 hr p.i. (Fig. 2).

3.3. Effects of Drugs on JEV viral RNA load
Treatment of Doxycycline has effect on viral RNA load. RNA load was reduced in 24 hour p.i. administered group. The effects of Doxycycline treatment were marginal at 48 hr and 96 hr p.i.. Treatment of Kanamycin had marginal effect on JEV load. (Fig. 3).

3.4. Effects of Drugs on Cytokine levels
In Doxycycline treated groups, the cytokines were expressed moderately at 24 hr p.i. The increased levels of IFN-γ and IL-6 following JEV infection was decreased after Doxycycline treatment at 24 hr p.i. The cytokines, especially IL-6 was expressed significantly in case of 96 hr p.i. In case of Kanamycin treated groups, Interleukin (IL) - 6 was the most expressed cytokine. The cytokines were expressed at low levels at 24 hr p.i. The cytokines, especially IL-6 was expressed highly in case of the 96 hr p.i. group (Fig. 4).

DISCUSSION

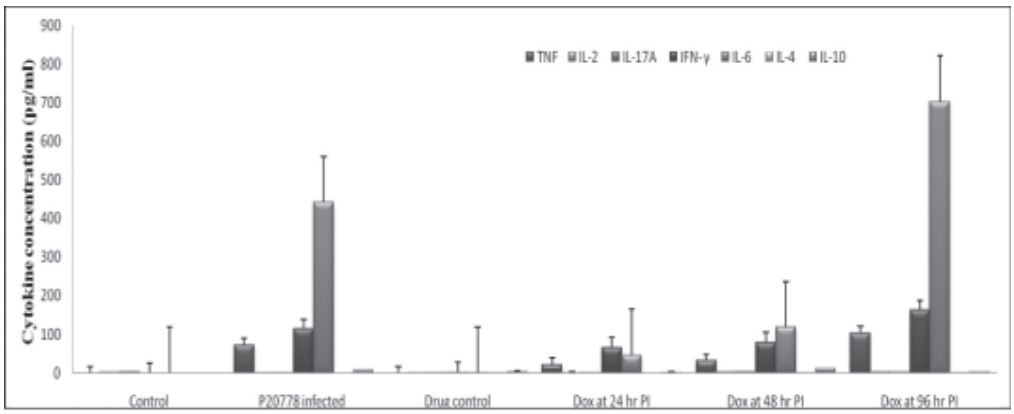
Treatment with Doxycycline at 50 mg/kg dose initiated 24 hr p.i. lowered the disease progression, prolonging the survival of the animals by a week. Titration of JEV in the harvested brains of the dead mice showed a marked reduction in plaque formation. It was observed that plaque formation was reduced at 24 hr p.i. (1×10^2 pfu/ml) compared to virus group (2×10^4 pfu/ml). However, a delay in drug administration to 48 hr and 96 hr p.i. negated this effect. The antiviral efficacy of drugs upon JEV load was assessed. Doxycycline treatment at 24 hr p.i. resulted in a decline in viral load. This effect of Doxycycline could be possibly due to the hydroxyl groups in tetracycline rigid skeleton. The hydroxyl groups are a source of reactive oxygen species (ROS) that irreversibly damage macromolecules such as DNA, RNA and proteins (Fuoco, 2012). Moreover, Doxycycline was able to moderately modulate the levels of pro-inflammatory cytokines at 24 hr p.i. treatment. The increased expression of pro-inflammatory cytokines (TNF, IFN-γ and IL-6) observed in virus infected group was decreased to a great extent after Doxycycline treatment in the 24 hr p.i. time period group. The present findings tallied with another study where Minocycline has been reported to decrease pro-inflammatory cytokines (TNF, IFN-γ and IL-6) at 24 hr p.i. (Mishra *et al.*, 2008). The expression of pro inflammatory cytokines after Doxycycline treatment at the two remaining groups of 48 hr p.i. and 96 hr p.i. increased. The cytokine IL-6 exhibited considerable up regulation in present findings. It is known to act both as proinflammatory and anti inflammatory cytokine. IL-6 has an important role in JEV infection as it increases blood brain barrier permeability. Microglial activation (the brain macrophage cells) leads to elevated levels of proinflammatory cytokine which includes IL-6 (Winter *et al.*, 2004; Larena *et al.*, 2011). Previous studies have shown that



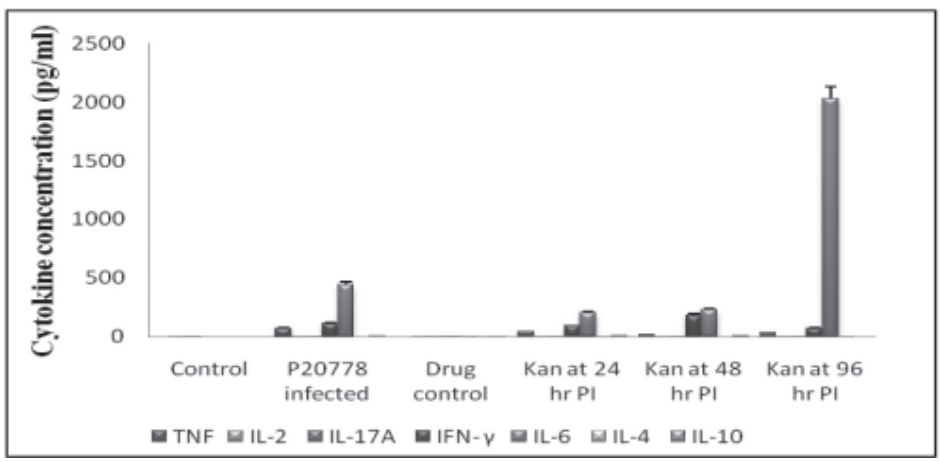
(a) Expression ratio of JEV viral load of groups treated with Doxycycline.

(b) Expression ratio of JEV load of groups treated with Kanamycin.

Figure 3. Expression ratio of JEV load of groups treated with (a) Doxycycline and (b) Kanamycin.



(a) Cytokine concentration (pg/ml) of experimental groups treated with Doxycycline.



(b) Cytokine concentration (pg/ml) of experimental groups treated with Kanamycin.

Figure 4. Cytokine concentration (pg/ml) of experimental groups treated with (a) Doxycycline and (b) Kanamycin.

Doxycycline reduces the secretion of IL-6 in stimulated cells administered 24 hr after infection with Lyme disease (Bernardino *et al.*, 2009). Therefore, it can be stated that Doxycycline modulated the proinflammatory cytokines to some extent at 24 hr p.i. treatment time period. Kanamycin was comparatively less effective. Thus, the present findings indicate that Doxycycline has an effective role in treatment as well as immunomodulatory behaviour *in vivo*. We also observed Doxycycline efficacy against JEV *in vitro* in our previous study. Antiviral activity was exhibited by Doxycycline with therapeutic index (TI) of 4.3. Treatment of cells reduced yield of JEV at sub cytotoxic dose. Thus it could be stated that effects of drugs were in direct action with the virus and not affecting the host cell membrane. The drug also declined viral RNA load in drug treated cells. Therefore, we concluded that doxycycline, a second generation tetracycline compound affected virion structure and altered replication causing inhibition of JEV induced pathogenesis *in vitro* (Topno *et al.*, 2016).

The present study indicated that Doxycycline delayed but did not prevent JE disease progression. The studies show a positive role of Doxycycline in treatment of JEV infection *in vivo*. The delay in the fatal outcome could be utilized for various

interventions to alter the end result in the patient. An aggressive symptom based treatment regime could reverse the condition of the patient, increasing the chances of survival. Hyperventilation and mannitol have been strongly advocated for controlling intracranial hypertension. Earlier studies have documented the beneficial role of Doxycycline in treatment of hospitalized randomized patients confirmed with Dengue fever. The cytokine levels significantly declined in the treated groups. The study concluded that Doxycycline may provide a clinical benefit in the treatment of Dengue by modulating the cytokine cascade (Castro *et al*., 2011). Therefore, the effects of Doxycycline administration in treatment of JE among hospitalized patients alone as well as in combination to mannitol and steroids merits study in near future. Moreover, Doxycycline is already a widely prescribed drug for human. It is on the World Health Organization's list of Essential Medicines, a list of the most important medications needed in basic health system. It is frequently used to treat Acne, Anthrax, Lyme disease, Rickettsial infections, chronic prostatitis, sinusitis, pelvic inflammatory disease and also used as prophylaxis against Malaria. However, prescription of tetracycline group of compounds entails a caution; it is contraindicated during pregnancy as well as in children up to eight years of age due to a potential for disrupting bone and tooth development.

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Conflict of Interests
None

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