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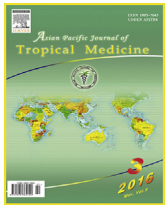

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Pharmacodynamics of aminoglycosides and tetracycline derivatives against Japanese encephalitis virus

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ABSTRACT

Objective: To explore the antiviral activity of antibiotic compounds, mainly aminoglycosides and tetracyclines against Japanese encephalitis virus (JEV) induced infection *in vitro*.

Methods: Antiviral activity were evaluated against JEV using cytopathic effect inhibition assay, virus yield reduction assay, caspase 3 level, extracellular viral detection by antigen capture ELISA and viral RNA levels.

Results: JEV induced cytopathic effect along with reduction of viral progeny plaque formation indicated antiviral potential of the compounds suggesting that antibiotics had broad spectrum activity. Doxycycline and kanamycin administration in dose dependent manner declined viral RNA replication.

Conclusions: The present study shows kanamycin and doxycycline can affect virion structure and alter replication causing inhibition of JEV induced pathogenesis *in vitro*.

1. Introduction

Japanese encephalitis (JE) is an arboviral neurologic disease of global public health importance. The disease is endemic in many parts of South Asia, Southeast Asia, East Asia and the Pacific. Most of JE disease is asymptomatic with no apparent illness. The ratio of asymptomatic to symptomatic infection varies between 25:1 and 1000:1 [1]. Case fatality rate among symptomatic patients is 25%–30%, with neuropsychiatric sequelae evident in 30%–50% of survivors [2]. Annually, approximately 30000–50000 JE cases, including 10000 deaths are reported [3]. JE is caused by a positive sense RNA virus–Japanese encephalitis virus (JEV), a member of JEV serocomplex of genus *Flavivirus* under family *Flaviviridae*. JEV is transmitted in humans mainly by *Culex* species of mosquitoes [4]. The treatment therapy for JE is mainly conservative and supportive.

Recently, tetracyclines and aminoglycosides specifically 2 deoxy streptamine (2 DOS) aminoglycoside derivative compounds have been successfully proved beneficial against viral infection namely dengue virus, West Nile virus and reovirus [5–7]. We report the efficiency of doxycycline and kanamycin against JEV induced infection in an *in vitro* pharmacokinetic model system.

2. Materials and methods

2.1. Biosafety statement

All infectious work with JEV was performed in a high containment facility at Arbovirology Laboratory, Regional Medical Research Centre (RMRC), Northeast Region, Indian Council of Medical Research, India. The present work was done under the Senior Research Fellowship scheme awarded by Indian Council of Medical Research, India.

2.2. Cell line, drugs and virus

Baby hamster kidney (BHK-21), a fibroblast cell line was procured from National Cell Centre for Sciences, Pune, India and maintained at RMRC, Northeast Region. The cell line was being maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 5% tryptose phosphate broth. Eight drugs were used in the present study: brefeldin A, chlortetracycline, demeclocycline, doxycycline, gentamicin, kanamycin, rolitetracycline and tetracycline.

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Ribavirin, a nucleoside analog metabolite compound and minocycline, a second generation tetracycline compound were used as control drugs. JEV strain P20778 (GenBank accession no. AF080251, source: human, year: 1958, place: Vellore, India) was obtained from National Institute of Virology, Pune, India and propagated in BHK-21 cell line.

2.3. Cytotoxicity of antibiotics and standards

Initially, cytotoxic concentration at 50% end point (CC_{50}) for the drugs were determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay [8,9]. Briefly, a 96 well plate (made by Genaxy) was pre-seeded with 3.5×10^5 cells/mL overnight. Next day, the MEM was discarded and replaced with respective dose concentration of drugs starting from 0 μg/mL to 520 μg/mL prepared in MEM. In control wells, plain MEM was added and allowed to incubate for 72 h (37 °C, 5% CO_2). After incubation, MTT reagent was added under dark conditions. Plate was again incubated for 4 h. After this, MTT solvent solution was added and incubated overnight. Following day, the reading of the plate was taken at 490 nm in an ELISA reader [10]. The optical density reading of the drugs at different concentrations was plotted. The concentration of compound reducing cell viability by 50% (CC_{50}) was determined from a curve relating percent cell viability to the concentration of compounds.

2.4. Screening for virus induced cytopathic effect inhibition (CPEI)

Since the two drugs, brefeldin A and demeclocycline, showed high cytotoxicity at very low doses, subsequent experiments were carried out with remaining drugs. Antiviral activity assay of drugs against JEV strain P20778 were screened using CPEI assay in a 96 well plate [8].

2.5. Confirmation of antiviral activity by virus yield reduction assay

The drug susceptibility was evaluated by virus yield reduction assay. Cells were pre-treated with different concentrations of drugs (0–100 μg/mL) for 24 h prior to infection. BHK 21 cells were inoculated with JEV strain P20778 at a multiplicity of infection of 0.1. Following a 1 h virus incubation period, the medium was removed and infected cultures were incubated with medium containing respective concentrations of drugs. At 48 h post-infection cultures were deep-frozen at −80 °C. After thawing of the cultures, infectious virus titres were determined for progeny virus yields by standard plaque titrations. All the experiments were run in triplicates. Percentage inhibitions of plaques were determined using the following formula:

% Inhibition = (Number of plaques in virus control − Number of plaques in drug treated)/(Number of plaques in virus control) × 100

The antiviral activity was expressed as 50% inhibitory concentration (IC_{50}) of the compound, *i.e.* concentration of the compound required to inhibit viral plaques by 50% as compared to virus control [6,8,11].

2.6. Extracellular antigen detection by Ag capture ELISA

An Ag capture ELISA was standardized to detect presence of antigens based on Gajanana *et al.*, 1995 with modifications [12].

2.7. Infectious virus yield inhibition by $TCID_{50}$/mL

The supernatant fluid was used to determine virus yield based on Reed and Munch method [13].

2.8. Caspase 3 assay: indicators of apoptosis activation

Caspase 3 of the samples was evaluated by using Caspase 3 Assay Kit, Colorimetric (made by Sigma–Aldrich company) according to manufacturer's instructions.

2.9. Monitoring of JEV RNA loads

JEV RNA was monitored using primers: F 5′-GGGAGTGATGGCCCCTGCAAAATT-3′ and R 5′-TCCAATGGAGCCAAAGTCCCAGGC-3′ [14]. The specificity of the amplicon was verified by melt curve analysis. Viral RNA load in antibiotics treated cells was compared to untreated controls and was normalized to the reference gene (*β-actin*). *β-Actin* gene primers for BHK-21 were: 5′-ACTGGCATTGTGATGGACTC-3′ and 5′-CATGAGGTAGTCTGTCAGGTC-3′ [15]. Data was expressed as relative fold expression to untreated controls, which was defined as 1.0 fold (100%). Triplicate reactions were carried out for each sample and no template control was included as a negative control.

2.10. Statistical analysis

Statistical analysis was done with the help of SPSS v.16 software. CC_{50} and IC_{50} values were calculated using means with standard deviations (SD). 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.

3. Results

3.1. Antiviral screening of drug efficacy

Initially, CC_{50} of all 10 drugs were determined (Table 1). Out of all drugs, both brefeldin A and demeclocycline showed

Table 1
CC_{50}, IC_{50} and TI dose of candidate compounds.

Candidate compound	CC_{50} (μg/mL)	IC_{50} (μg/mL)	TI
Brefeldin A	10 ± 2	–	–
Chlortetracycline	189 ± 8	50 ± 7	4.0
Demeclocycline	9 ± 2	–	–
Doxycycline	95 ± 6	22 ± 1.2	4.3
Gentamicin	109 ± 12	53 ± 9	2.0
Kanamycin	381 ± 139	70 ± 15	5.4
Rolitetracycline	300 ± 77	76 ± 25	4.0
Tetracycline	303 ± 139	74 ± 33	4.0
Standard			
Minocycline	342 ± 17	34 ± 9	10.0
Ribavirin	195 ± 4	20 ± 2	10.0