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In-vivo assessment of the effects of trypanocidal drugs against *Trypanosoma evansi* isolates from Philippine water buffaloes (*Bubalus bubalis*)

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ABSTRACT

The effects of the trypanocidal drugs against *Trypanosoma evansi* isolated from Philippine water buffaloes from the three island groups were comparatively evaluated. Specifically, the study determined the duration of the efficacy, relapse and death per drug dosage using laboratory mice. A total of 270 inbred Balb/c mice were divided into three groups corresponding to the three trypanosome isolates (Luzon, Visayas, and Mindanao). Each group had three sets, corresponding to the three trypanocidal drugs used with five treatment levels, and one control group each. Each experimental group was composed of five mice. Each mouse was inoculated with 0.2 mL of *T. evansi* intraperitoneally and blood was examined under the microscope. Parasitemia level was determined using the "Rapid Matching Method". Effective and curative doses were noted and evaluated through t-test and bio-assay graphical analyses. Results showed that the Luzon isolate was sensitive to ≥5 mg/kg of diminazene diaceturate and ≥10 mg/kg of both isometamidium chloride and quinapyramine sulphate + chloride. The Visayas isolate was sensitive to ≥5 mg/kg, ≥10 mg/kg, and ≥3 mg/kg of diminazene diaceturate, isometamidium chloride and quinapyramine sulphate + chloride, respectively. The Mindanao isolate was sensitive to ≥3 mg/kg of diminazene diaceturate and quinapyramine sulphate + chloride and 20 mg/kg of isometamidium chloride. The study suggested diminazene as the recommended drug against Luzon isolates, quinapyramine against Visayas isolates and either diminazene or quinapyramine against Mindanao isolates.

Key words: drug sensitivity, trypanocidal drugs, *Trypanosoma evansi*, Philippines

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Introduction

Surra is an important disease of farm animals such as cattle and water buffaloes in the Philippines. In 1990-1998, it produced economic losses amounting to P44.8 million (US$ 1.15 million) due to death, excluding losses from reduced reproductive performance, milk yield, loss of weight, and draught power, which are likely to be substantially greater (MANUEL, 1998; VENTURINA et al., 2008).

Surra has been reported to be endemic in the three major island groups in the Philippines. Recently, a study showed differing virulence of the three *Trypanosoma evansi* (*T. evansi*) isolates in the country, tagged as Luzon, Visayas and Mindanao isolates (VERDILLO et al., 2012). It was then hypothesized that the diversity of the three isolates might also result in a difference in drug response. Numerous drug treatments have been tested over the years, but have not been widely adopted because of various problems associated with their efficacy, availability, toxicity and/or affordability (VENTURINA et al., 2008). In the Philippines, the commonly used trypanocidal drugs are naganol, diminazene, suramin, isometamidium, quinapyramine and cymelarsan (VENTURINA et al., 2008).

Sensitivity to trypanocidal drugs means effectiveness against the target protozoa. However, prolonged use of these agents may lead to the development of resistance. Determination of trypanocidal sensitivity or resistance of trypanosomes isolated in water buffaloes per island group will help veterinarians for better management and strategic treatment of Surra in the country.

The objective of the present study was to compare trypanocidal effects of selected drugs against *T. evansi* isolated from Philippine water buffaloes, representing the three different island groups.

Materials and methods

Two hundred seventy (270) Balb/c mice (7-8 weeks of age and weighing about 25 to 30 grams) were used and divided into three groups corresponding to the three trypanosome isolates (Luzon, Visayas, and Mindanao). Each group had three set-ups, corresponding to the three drugs, 7% diminazene diaceturate (Sequent, India), 2% isometamidium chloride (Merial, France) and 16.7% quinapyramine sulphate and chloride (Cipla, India), with five mice per treatment and control groups.

The experiment conforms to the guidelines for care and use of laboratory animals, published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996)

Viability testing. Blood with trypanosomes was preserved at -80 ºC as a 1:1 mixture with bicine buffered saline (bbs) solution (pH 8.0) plus 20% w/v of glycerol and 10% v/v heparin. Samples were taken from the deep freezer and thawed in a water bath (37 °C

for 15 minutes). A motility test for the protozoa was undertaken by placing a tuberculin syringe-aspirated drop-sized blood sample, of sufficient quantity to spread and cover the entire interface between a glass slide and a 24 × 24 mm cover slip. It was examined under 40 × 10 magnification.

Quantification of trypanosomes. If they were motile, 0.2 mL was inoculated intraperitoneally per mouse per isolate. Three days post-inoculation, a small drop of blood was collected from the tail of the inoculated mouse and placed on a glass slide, with a 24 × 24 mm coverslip, and examined under 40 × 10 magnification. If the parasitemia level of the inoculated mouse attained a log of 9.0, the mouse was sacrificed and 1 mL of blood was collected intracardiac using tuberculin syringe. The collected blood was placed in a microcentrifuge tube and diluted by adding a drop of bbs. After every addition of bbs, the sample was mixed gently. Dilution was stopped only when 32 trypanosomes per field (log of 8.1) were attained. This corresponds to 126,000,000 trypanosomes per 1.0 mL dilution (HERBERT and LUMSDEN, 1976). The experimental inoculum size of 0.2 mL was estimated to contain 25,200,000 organisms. Preserved cultures with the first and second passages were used in the study. Passage in the mice was restricted to two to three times, in order to minimize selection of sub-populations from the original isolates (EISLER et al., 2001).

Inoculation of trypanosomes, drug regimen and checking the parasitemia. On the first day, each experimental mouse was weighed prior to the designation of markers and dosage computations and the prepared 0.2 mL sample was inoculated by intraperitoneal route (IP). At 24 hours post-inoculation (2nd day), parasitemia in tail blood was checked from each experimental animal.

Drug injection was by IP route, each treatment group received the desired dosage regimen (the weight of the animal multiplied by the experimental dose and divided by the drug concentration), while the control groups received 0.2 mL distilled water, on the same occasion. The five treatments were as follows: 1 mg/kg, 3 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg for diminazene and isometamidium, and 15 mg/kg for quinapyramine. The computed dose was diluted with 0.2 mL distilled water in a microcentrifuge tube before it was injected intraperitoneally.

Parasitemia was checked at 12, 24, 48 and 72 hours post-treatment and subsequently twice a week until the end of a 60-day observation period. The number of days from the first detection and description of parasitemia was recorded. Charts and tables from the rapid matching method for estimating parasitemia by HERBERT and LUMSDEN (1976) were used in the study.

Animal identification. Body markings and codes were made to avoid errors in data recording and ensure accurate and comprehensive analysis. Different anatomical areas were coded by applying picric acid solution using dipsticks with cotton.

Data interpretation. The number of cured animals was used for interpretation of the results. Curative Dose 80 and 100 (CD_{80}; CD_{100}) indicated that the drug dosage was effective if at least four out of the five treated mice were cured after the period of 60 days. Hence, the isolates treated were said to be sensitive. If fewer than four mice were cured (CD_{60}, CD_{40}, CD_{20}, or CD_{0}), the drug dosage was ineffective. Hence the isolate treated was said to be tolerant (EISLER et al., 2001). The use of five mice per group allowed an easy calculation of the values (SONES et al., 1988).

Data analyses. Comparisons of effective dose and curative dose values were done using bio-assay graphical analysis. T-test was used to compare the difference between the mean days of death from the control group against non-curative drug dosage.

Results

Effects of the three trypanocidal drugs against the different isolates. The effects of each drug dosage varied among the three trypanosome isolates. The result shows that the higher drug dosages were curative.

The effects of the three trypanocidal drugs against the three trypanosome isolates are shown in Fig. 1. Luzon isolates were sensitive at 5 mg/kg with CD_{100} in diminazene, and at 10 mg/kg with CD_{100} and CD_{80} in isometamidium, and quinapyramine, respectively.

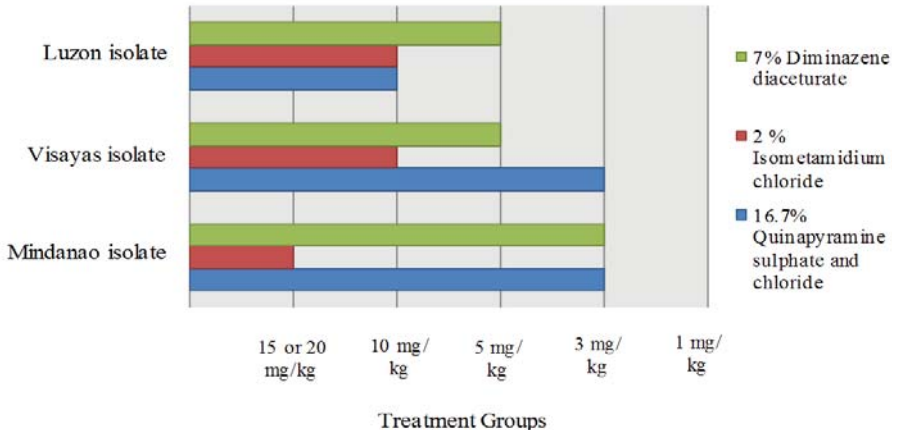

Fig. 1. Bio-assay graphical analysis comparing the different trypanocidal dosages with CD_{80} and CD_{100} after 60 days against *T. evansi* isolated from Philippine water buffaloes

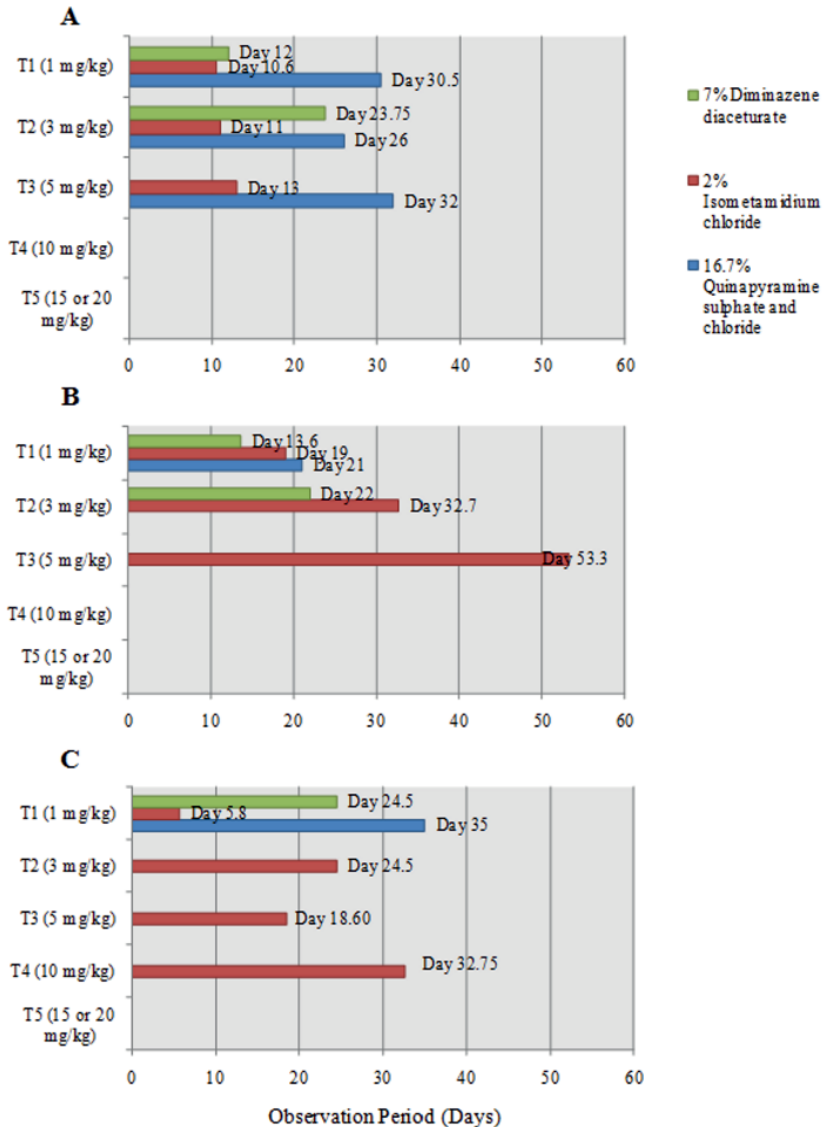

Fig. 2. Bio-assay graphical analysis comparing the onset of death after relapse per drug dosage in (A) Luzon, (B) Visayas, and (C) Mindanao isolates

Visayas isolates were sensitive beginning at 5 mg/kg with CD_{80} in diminazene, at 10 mg/kg with CD_{100} in isometamidum and at 3 mg/kg with CD_{100} in quinapyramine. Mindanao isolates were sensitive beginning at 3 mg/kg with CD_{100} in diminazene, at 20 mg/kg with CD_{100} in isometamidium and at 3 mg/kg with CD_{80} in quinapyramine.

Evaluation of the drug dosage that showed relapse and death using the curative dose (CD) values. Fig. 2-A shows the onset of death after relapse per dosage in Luzon isolates. In diminazene, T1 and T2 showed CD_0 at mean of Day 12 and 23.75, respectively. The latter was highly significant compared to the control groups ($P<0.0000$ at $P<0.05$). In isometamidium, T1, T2 and T3 showed CD_0, CD_{20}, and CD_{60} days at mean of Days 10.6, 11 and 13, respectively. In quinapyramine, T1, T2 and T3 showed CD_{20}, CD_0, and CD_{20} at Days 30.5, 26 and 32, respectively.

The onset of death after relapse per dosage in Visayas isolates is shown in Fig. 2-B. In diminazene, T1 and T2 showed CD_0 and CD_{60} days at mean of Days 13.6 and 22, respectively. In isometamidium, T1, T2 and T3 showed CD_{40} (both T1 and T3), and CD_{20} at Days 19, 32.7 and 53.3, respectively. In quinapyramine, T1 showed CD_{20} at Day 21.

Fig. 2-C shows the onset of death after relapse per dosage in Mindanao trypanosome isolates. In both diminazene and quinapyramine, T1 showed CD_{20} and CD_{60} days at mean days 24.5 and 35, respectively. In isometamidium, T1, T2, T3 and T4 showed CD_0 (both T1 and T3) and CD_{20} (both T2 and T4) at days 5.8, 24.5, 18.60 days, and 32.75, respectively. However, onset of death was not significant in T1 ($P<1.0000$) and T2 ($P<0.1002$) while highly significant at T3 ($P<0.0045$) to T4 ($P<0.0017$) compared to the control group (significantly different at $P<0.05$).

Discussion

The isolates in this study were obtained from infected water buffaloes in different areas that had received no trypanocidal treatment. Drug efficacy and failure to treat a certain disease are influenced by numerous factors. The results obtained could be influenced by some mechanisms concerning the drugs, the parasites, and the hosts (GEERTS and HOLMES, 1998).

One factor that could influence the results are the different modes of action of the trypanocidal drugs against the target parasites. Diminazene disrupts the phosphoglycerate synthesis by binding to the DNA (ADAMS, 1995; MEDINA, 2008). The drug selectively blocks the replication of kinetoplast DNA (MAMMAN et al., 1994). Isometamidium cleaves the kinetoplast DNA topoisomerase complexes. The trypanosome kinetoplast is the primary site of isometamidium accumulation (GEERTS and HOLMES, 1998; ANONYM., 2008). It inhibits DNA- primed RNA polymerase as a result of intercalation of the drug with DNA. This causes local unwinding and lengthening of the DNA helix and thus interferes with its function as a primer in nucleic acid synthesis (ADAMS, 1995; MEDINA,

2008). Quinapyramine targets protein synthesis by kDNA condensation with extensive loss of ribosomes (MEHLHORN, 2008).

The three drugs target the kinetoplast of the organism. However, some studies have reported the absence of kinetoplast in some strains of *T. evansi.* These dyskinetoplastic forms were found in wild strains that have undergone mutation, isolated from animals that received treatment with trypanocides (BRUN et al., 1998; ZWEYGARTH et al., 1990). This was also observed by JUYAL (Newer perspective in the diagnosis and control of Trypanosomosis (Surra) in domestic livestock in India, 2002) in his study, that dyskinetoplastic forms of *T. evansi* appear after treatment with a variety of trypanocidal drugs, including diminazene and pyrithidium. Therefore, relapse of infection by the organisms could occur because the drugs were not able to target the relevant anatomical part and death occurred in the mice. In cases where resistance is not a problem, one possible reason for relapse could be attributed to the inaccessibility of the drugs to reach the tissue stages of trypanosomes (JENNINGS et al., 1977; AL-MOHAMMED, 2008). Trypanosomes reside in the blood vessels of all organs, in the extravascular spaces of the brain, and the interstitial tissues of the lungs and testes (SUDARTO et al., 1990). Among these structures, the brain is the site where diminazene in particular was not able to penetrate (PEREGRINE and MAMMAN, 1993). This could be the reason why some trypanosomes escaped from the trypanocidal action of diminazene, in which relapse of infection is possible. This finding is also observed in the case of *Trypanosoma brucei* infection after relapse had occurred from a privilege site inaccessible to the diminazene (MAMMAN et al., 1994; ONYEYILI and EGWU, 1995; NWIYI et al., 2006).

Extensive use of the limited number of commercially available trypanocides has resulted in the appearance of trypanosome strains resistant to the drugs (MBWAMBO et al., 1988; JENNINGS, 1990; FAIRLAMB, 1991; ELRAYAH and KAMINSKY, 1991; ZHANG et al., 1993). It could be that isometamidium was one of the frequently used anti-trypanosomes in the Mindanao area. As the results revealed, the drug needed the highest dosage (20 mg/kg) to cure the mice infected with trypanosome isolated from the island, as compared to the curative dosage (10 mg/kg) needed against trypanosome isolated from Luzon and Visayas islands. Diminazene was also used in the country and traded under different names. Based on the results, the drug needed a 10 mg/kg dosage to cure infected mice, which is higher in comparison with the recommended dosage of 3.5 mg/kg in large animals. This could be the reason why some reports stated that one or two replication sites revealed *T. evansi* isolates to be resistant, from the three provinces in Visayas, such as Northern Samar (83.3%), Leyte (6.6%) and Biliran (100%) in which diminazene has been used for more than nine years (VENTURINA et al., 2008).

Dosages that were found curative in this study could be compared to the reports made by ZHANG et al. (1991). The *T. evansi* used by the latter was taken from infected horses

in 1982 and was not isolate specific. They observed that it was sensitive at 9 mg/kg in diminazene, which is in the range of the drug's curative dosage, from 3 to 20 mg/kg based on the results. In isometamidium, they observed that a dosage of more than 20 mg/kg was needed, while 10 to 20 mg/kg were already curative in this study. In quinapyramine, it was found that a dosage at 1.25 mg/kg was already curative in the mice. But, the results of this study showed that higher dosages from 3 to 15 mg/kg were needed to eliminate the trypanosomes.

The effects of these trypanocidal drugs were also studied. Diminazene failed to control infection in 20% of mice separately infected with two Mindanao isolates (DARGANTES, 2010). Isometamidium given intraperitoneally at dose rates ranging from 2-15 mg/kg body mass provided weak chemotherapeutic activity in cats experimentally infected with the Philippine strain of *T. evansi,* as studied by MANUEL (1985). A quinapyramine dose rate of 1 mg/kg was able to cure all four mice used in the experiments (GILLINGWATER et al., 2009).

Relapse of infection and death were also seen by some authors in their experiments. DARGANTES (2010) claimed that treatment with diminazene was found to be ineffective because relapse occurred on the 27th day post-treatment when used in rats and goats. DIVINA (2004) reported a reoccurrence of parasitemia in mules and horses after five days of treatment in severely parasitized animals. Preliminary studies at Central Mindanao University showed that diminazene diaceturate (the standard treatment for Surra in the Philippines) did not cure *T. evansi* infection in goats when a single dose of 7 mg/kg was given (REID, 2001).

The results were comparative with the recommended dosages used to treat Surra in large animals. Diminazene is recommended at a dosage of 3.5 mg/kg. In the study, 5 mg/kg was needed to cure infection by Luzon and Visayas isolates, but 3 mg/kg against Mindanao isolates. Isometamidium is recommended at 0.25 to 0.50 mg/kg. In the study, 10 mg/kg was needed to cure infection against Luzon and Visayas isolates, but 20 mg/kg against Mindanao isolates. Quinapyramine is recommended at a dose rate of 4.4 mg/kg dose rate. Meanwhile, 10 mg/kg was needed to cure infection by Luzon isolate while 3 mg/kg against Visayas and Mindanao isolates.

Conclusion

The effects of the three trypanocidal drugs (7% diminazene diaceturate, 2% isometamidium chloride, 16.7% quinapyramine sulphate + chloride) varied among the three Philippine water buffaloes *Trypanosoma evansi* isolates (Luzon, Visayas, and Mindanao) in murine model. Based on the findings, the study suggests diminazene as the

recommended drug against Luzon isolates, quinapyramine against Visayas isolates and either diminazene or quinapyramine against Mindanao isolates.

The determined minimum curative doses in experimental mice did not exceed the dose rates indicated for large animals. Except for the 2% isometamidium chloride, in which the recommended dosage at 1 mg/kg is no longer effective against the Mindanao isolate. The dose should be increased to 2 mg/kg.

Moreover, it is suggested that a continuous study should be conducted to investigate presence of different or new isolates in each island groups and evaluate their trypanocidal sensitivities.

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SAŽETAK

Istraženi su učinci tripanocidnih lijekova na izolate vrste *Trypanosoma evansi* izdvojene iz triju otočnih skupina filipinskih vodenih bivola. Određivano je trajanje učinkovitosti, pojava recidiva i uginuća u laboratorijskih miševa. Ukupno je 270 miševa Balb/c bilo podijeljeno u u tri skupine što je bilo sukladno trima izolatima s tri filipinska otoka i to Luzon, Visayas i Mindanao. Svaka skupina bila je podijeljena u tri podskupine koje su peterokratno dobivale tri različita lijeka. Unutar svake skupine određena je i kontrolna podskupina. U svakoj pokusnoj skupini bilo je pet miševa. Svakom mišu bila su intraperitonejski ubrizgana 0,2 mL kulture *T. evansi* i izvađena krv za mikroskopsku pretragu. Razina parazitemije određivana je brzom metodom (engl. rapid matching method). Učinkovitost i ljekovita doza vrednovane su *t*-testom i grafičkom analizom biološkog pokusa. Rezultati su pokazali da je izolat Luzon bio osjetljiv na ≥5 mg/kg diminazenova diaceturata i ≥10 mg/kg izometamidijeva klorida i kombinacije kvinapiramin sulfata i klorida. Izolat Visayas bio je osjetljiv na ≥5 mg/kg diminazen diaceturata, ≥10 mg/kg izometamidij klorida i ≥3 mg/kg kombinacije kvinapiramin sulfata i klorida. Izolat Mindanao je bio osjetljiv na ≥3 mg/kg diminazen diaceturata i kombinacije kvinapiramin sulfata i klorida, te 20 mg/kg izometamidij klorida. Može se reći da je diminazen bio učinkovit na

izolate s otoka Luzon, kvinapiramin na one s otoka Visayas, dok su diminazen ili kvinapiramin bili učinkoviti na izolate s otoka Mindanao.

Ključne riječi: osjetljivost, tripanocidni lijekovi, *Trypanosoma evansi*, vodeni bivol, Filipini