Original Article

Antiviral Activity of Four Commercial Tilmicosin Preparations against Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): An *In Vitro* Study

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Abstract

The efficacy of four commercial tilmicosin preparations (A, B, C and D) against type 2 porcine reproductive and respiratory syndrome virus (PRRSV), 01NP1 (Thai isolate), infection in cultured pulmonary alveolar macrophages (PAMs) was conducted *in vitro*. Primary PAMs were collected from four 4-week-old PRRSV-free pigs. After separately plated onto four 24-well plates, PAMs were treated separately by 4 commercial tilmicosin preparations of 2 concentrations each (0.1 mg/ml and 0.01 mg/ml). The treated PAMs were inoculated with 0.05 MOI of 01NP1 strain and were stained with PRRSV specific antibody using immunoperoxidase monolayer assay (IPMA) to evaluate the quantity of PRRSV infected cells after 12 hours post infection (HPI). Comparing to the untreated-tilmicosin PRRSV-infected PAMs, all tilmicosin-treated preparations exhibited significant virus titer reduction against 01NP1. Based on the results, 0.01 mg/ml of tilmicosin B solution exhibited the greatest PRRSV-titer reduction (65%), but was not statistically different from the others. The results indicated that tilmicosin could be one of the effective chemotherapy in reducing type 2 PRRSV infection *in vitro* regardless of differences in the preparations. The information obtained is of interest for practitioners for future study of implementation of tilmicosin use in PRRSV-positive farms.

Keywords: antiviral activity, *in vitro*, PAMs, PRRSV, tilmicosin

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most problematic infectious diseases in the swine industry, caused by PRRS virus (PRRSV). The disease is currently spreading worldwide causing major impacts on both microeconomic and macroeconomic levels. Direct effects of the disease include weight loss, poor growth performance and reproductive failure (Pejsak et al., 1997). Moreover, interleukin (IL)-10 induced by PRRSV infection is known as immunological interference in infected pigs (Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003) causing a higher risk of secondary infections. According to the negative effects of the disease, PRRS has become one of the trade barriers for live pigs and swine products in many countries around the world (Zimmerman, 2008).

When encountered with PRRSV outbreaks, traditional prevention and control strategies such as management, biosecurity and vaccination are implemented. However, those strategies are hampered by immune evasion strategies and various antigenic heterogeneities of the causative viruses (Murtaugh et al., 2002). IL-10 induced by the nucleocapsid protein of the virus is one of the major immune evasion mechanisms of the virus. The antigenic heterogeneities and the immune-inhibitory effects of PRRSV are the reasons why there are no available PRRSV vaccines inducing fully cross-protection against all PRRSV strains (Thanawongnuwech and Suradhat, 2010).

With limited successful controlling procedures, eradication is an alternative to eliminate PRRSV from the infected herd. The eradication procedures including depopulation and repopulation are very costly and complicated in high pig density areas (Thanawongnuwech and Suradhat, 2010). For these reasons, many alternative ways to control the virus in the field have been suggested including chemotherapeutic use.

Tilmicosin is a tylosin derivative macrolide antibiotic. This drug is recommended for treatment and prevention of respiratory disease associated with bacterial infection in bovine and ovine species. It also has been proven as an effective chemotherapy against virus including PRRSV *in vitro* (Du et al., 2011). Similarly, based on an *in vivo* study, tilmicosin has been extensively approved that this drug can induce positive impacts in PRRSV infected herds (Misener et al., 2006). However, in one experiment, tilmicosin did not exhibit the antiviral effect in PRRSV infected pigs (O'Sullivan et al., 2012) and the antiviral activity of tilmicosin against PRRSV is still debatable.

In addition, there are many commercially available tilmicosin products. However, the most effective concentration of each commercial tilmicosin preparations is still in doubt. This information is needed for swine practitioners to select the most suitable product in case of PRRSV infection.

In this study, antiviral activity of 4 selected commercial preparations of tilmicosin (A, B, C and D) at 2 concentrations, 0.1 mg/ml and 0.01 mg/ml, was compared. Using primary cultures of pulmonary alveolar macrophages (PAMs) from 4 pigs, performances of these selected 8 tilmicosin solutions against 0.05 MOI of 01NP1, a Thai isolate type 2 PRRSV (Amonsin et al., 2009), were tested and evaluated.

Materials and Methods

Tilmicosin and RPMI preparation: In this study, 4 selected tilmicosin preparations (A, B, C and D) were obtained commercially. Each one was prepared by diluting in Rosswell Park Memorial Institute medium (RPMI) to the concentration of 0.1 mg/ml, as A0.1, B0.1, C0.1 and D0.1. Similarly, these 4 tilmicosin preparations were diluted into the concentration of 0.01 mg/ml, as A0.01, B0.01, C0.01 and D0.01, respectively. All 8 tilmicosin solutions were prepared and kept in 4 ^{o}C until used in the same day.

Virus preparation: Type 2 PRRSV, 01NP1 (a Thai isolate), was kindly provided by the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). The stock virus (10^5 $TCID_{50}$/ml) was diluted in each tilmicosin containing-RPMI solution (A0.1, B0.1, C0.1, D0.1, A0.01, B0.01, C0.01 and D0.01) to obtain the concentration of 0.05 MOI.

Macrophage collection and culture: For 4 repeatedly experiments, pulmonary alveolar macrophages (PAMs) used in this study were obtained from 4 PRRSV-free pigs (kindly provided by Charoen Pokphand Foods PCL). The experimental pigs were euthanized at 4 wk of age by intravenous injection with an overdose of barbiturates (sodium pentobarbital, 20%) under the permission of Chulalongkorn University Animal Care and Use Committee (CU-IACUC Animal Use Protocol Number: 13310019). Lung of each pig was used for sterile bronchoalveolar lavage for PAMs collection. PAMs were collected in 25 ml sterile phosphate buffered saline (PBS) solution counted and kept separately. One milliliter (10^6 cells per ml) of PAMs from each pig was cultured in each 24 well cell-culture plates. Eight wells of each plate were treated as negative and positive control wells equally. The negative control wells contained PAMs and RPMI without tilmicosin added, whereas the positive control wells contained PAMs with 0.05 MOI of 01NP1 in RPMI without tilmicosin. Briefly, after seeding for 2 h, PAMs in each treatment group were pre-treated with different 8 tilmicosin solutions as mentioned above for 6 h. Afterwards, the cells in each well were inoculated with 0.05 MOI of 01NP1. Subsequently, after 2 h of the inoculation, the same RPMI solutions of each well were added respectively for a final volume of 1 ml and were later incubated for 11 h. Finally, PAMs in each well were fixed with 40% formalin solution before immunoperoxidase staining for PRRSV antigen detection using SDOW-17 monoclonal antibody (Rural Technologies Inc., Brookings, South Dakota). Numbers of PRRSV infected cells were randomly counted manually out of 200 cells in each well, averaged and compared to the mean positive control groups to evaluate the antiviral activity percentage of each tilmicosin solution.

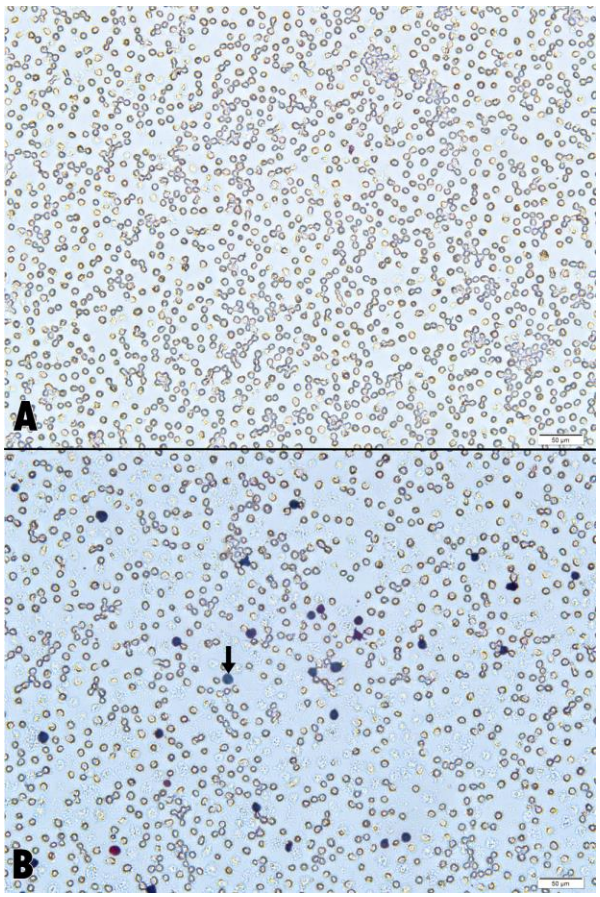

Figure 1 (A) Negative PRRSV-specific immunoperoxidase staining **(B)** Positive PRRSV-specific immunoperoxidase staining on PAMs (arrow)

Statistical analysis: Data were analyzed using one-way ANOVA to evaluate percent reduction in PRRSV infected cells among each treatment group compared to the positive control group. Moreover, data were analyzed by Dunnett's test for further multiple comparisons.

Results

Since PAMs in each well were stained with PRRSV specific-immunoperoxidase staining, the infected positive cells were characterized as a brownish-black or reddish staining, located in the cytoplasm of PRRSV-infected PAMs (Fig 1).

To evaluate the percent reduction after treatment of each group, percentages of PRRSV-positive staining cells of each treatment group were compared to the results of the positive control group. The result of each treatment is demonstrated in Table 1. The averages of percent reduction in A0.1, A0.01, B0.1, B0.01, C0.1, C0.01, D0.1 and D0.01 were 64.19%, 48.27%, 45.49%, 65.62%, 47.11%, 58.75%, 46.04% and 50.80%, respectively (Fig 2).

According to the statistical tests, all tilmicosin solutions had significant reduction effects against the Thai type 2 PRRSV (01NP1) infection compared to the positive control group with no medication ($p<0.05$). The treatment group exhibiting the highest percent reduction (65.62%) was B0.01 containing 0.01 mg/ml of tilmicosin B solution.

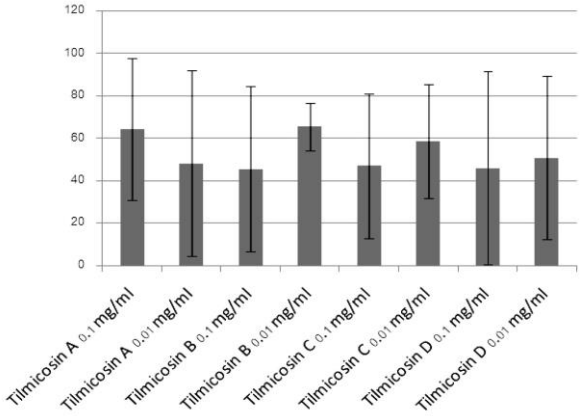

Figure 2 Average percent reduction in PRRSV-positive PAMs when inoculated with Thai Type 2 PRRSV (01NP1) at 0.05 MOI on treated 8 tilmicosin preparations

Table 1 Average percentages of positive PAMs inoculated with Thai Type 2 PRRSV (01NP1) at 0.05 MOI on treated commercial tilmicosin (A-D)

Groups	Concentration (mg/ml)	Mean ± SD
Positive control		2.38±0.86
tilmicosin A	0.1	1.06±1.30*
	0.01	1.50±1.68*
tilmicosin B	0.1	1.54±1.4*
	0.01	0.82±0.43*
tilmicosin C	0.1	1.44±1.19*
	0.01	1.15±1.08*
tilmicosin D	0.1	1.57±1.74*
	0.01	1.42±1.54*

*significantly different ($p<0.05$) compared to the positive control group

Discussion

The results from the present study demonstrated that all tilmicosin preparations tested in this study had variation on viral reduction activity when compared to previous studies (Du et al., 2011). Many factors may involve in the variation including different PRRSV genotypes (Allende et al., 1999; Dea et al., 2000) or isolates (Yoshii et al., 2005), individual drug metabolism (Meyer and Zanger, 1997) and also duration of the treatment (Brown and Nathwani, 2005). Other factors need further evaluation to demonstrate the efficacy of tilmicosin against PRRSV in different cases.

At present, knowledge about antiviral mechanism in animals is still limited. Antiviral agents previously studied in swine diseases include viral polymerase inhibitors (Vrancken et al., 2008, 2009a,b), bacterial topoisomerase inhibitors (Mottola et al., 2013) and herbal extracts (Gao et al., 2013; Kwon et al., 2013). Each product has its advantages and disadvantages with the consideration of antiviral efficiency, mechanisms and cost/effectiveness when used in commercial swine herds. Based on the results from this study, tilmicosin is a good candidate for future application for PRRSV control in PRRSV-positive farms. It has both effects of bacterial protein

biosynthesis inhibition and antiviral effects shown *in vitro*. Previously, tilmicosin demonstrated the antiviral mechanism most likely related to altered pH in the endosome of the cells. This mechanism produces an effect on disturbance of endosomal pH and ion-channel activity on the viral membrane (Du et al., 2011). However, the current knowledge of this drug is still limited and in need of further investigations to improve the drug efficiency and also for further applications in the field.

In conclusion, this *in vitro* study provides the knowledge about the antiviral activities of tilmicosin against 01NP1, a Thai type 2 PRRSV isolate. It is suggested that tilmicosin is a novel alternative chemotherapy and might be an alternative application in the case of PRRSV infection in swine as well as for the reduction in complication from the secondary respiratory bacterial infections. However, further study is needed for an application in farm level especially the mechanism of tilmicosin against PRRSV infection.

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บทคัดย่อ

การศึกษาประสิทธิภาพของยาปฏิชีวนะ tilmicosin 4 ชนิดต่อการติดเชื้อไวรัสพีอาร์อาร์เอสในห้องปฏิบัติการ

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ศึกษาประสิทธิภาพของสารละลายยาปฏิชีวนะทิลมิโคซินที่มีขายในเชิงพาณิชย์ 4 ชนิด ได้แก่ A, B, C และ D ต่อการป้องกันการติดไวรัสพีอาร์อาร์เอสเข้าสู่เซลล์มาโครฟาจในปอดหรือ pulmonary alveolar macrophage (PAMs) ซึ่งเป็นเซลล์เป้าหมายของไวรัส โดยใช้ไวรัส 01NP1 เป็นตัวแทนของไวรัสพีอาร์อาร์เอส กลุ่มสายพันธุ์อเมริกาเหนือที่พบในประเทศไทย ทำการเพาะเลี้ยงเซลล์มาโครฟาจในปอดที่ได้จากสุกรที่ปลอดไวรัสพีอาร์อาร์เอสอายุ 4 สัปดาห์ จำนวน 4 ตัว ในถาดเลี้ยงเซลล์ชนิด 24 หลุม จำนวนทั้งหมด 4 ถาด ทำการเตรียมเซลล์ในกลุ่มทดลองด้วยสารละลายยาปฏิชีวนะทิลมิโคซิน 4 ชนิดที่มีความเข้มข้น 2 ระดับ ได้แก่ 0.1 มิลลิกรัม/มิลลิลิตรและ 0.01 มิลลิกรัม/มิลลิลิตร ตามลำดับ จากนั้นทำการเพาะเชื้อไวรัสพีอาร์อาร์เอสที่มีความเข้มข้น 0.05 MOI ในแต่ละหลุม หลังจากเพาะเชื้อไวรัสเป็นเวลา 12 ชั่วโมง จึงทำการย้อมเซลล์มาโครฟาจในปอดในแต่ละหลุม โดยอาศัยแอนติบอดีที่จำเพาะต่อไวรัสพีอาร์อาร์เอสด้วยวิธี immunoperoxidase monolayer assay (IPMA) เพื่อประเมินจำนวนเซลล์มาโครฟาจในปอดที่ติดไวรัสพีอาร์อาร์เอสเปรียบเทียบระหว่างกลุ่มทดลองและกลุ่มควบคุมที่ปราศจากยาปฏิชีวนะทิลมิโคซินทั้ง 4 ชนิด จากการทดลองพบว่าในทุกกลุ่มทดลองที่ได้รับสารละลายยาปฏิชีวนะทิลมิโคซินมีการติดไวรัสพีอาร์อาร์เอส 01NP1 ในเซลล์มาโครฟาจในปอดลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุม โดยเฉพาะยาปฏิชีวนะทิลมิโคซินกลุ่ม B ที่ความเข้มข้น 0.01 มิลลิกรัม/มิลลิลิตร ซึ่งแสดงผลในการลดการติดไวรัสได้ดีที่สุด โดยสามารถลดการติดไวรัสได้ร้อยละ 65 เมื่อเปรียบเทียบกับกลุ่มควบคุม ข้อมูลดังกล่าวเป็นหลักฐานแสดงว่ายาปฏิชีวนะทิลมิโคซินมีแนวโน้มว่าสามารถใช้เพื่อลดการติดไวรัสพีอาร์อาร์เอสกลุ่มสายพันธุ์อเมริกาเหนือได้ในห้องปฏิบัติการ ซึ่งอาจสามารถนำไปศึกษาประยุกต์ใช้เพื่อลดความเสียหายจากการติดเชื้อไวรัสพีอาร์อาร์เอสในฟาร์มสุกรในอนาคตเมื่อมีการศึกษาเพิ่มเติมต่อไป

คำสำคัญ: ประสิทธิภาพการต่อต้านไวรัส การทดลองในห้องปฏิบัติการ เซลล์มาโครฟาจในปอด ไวรัสพีอาร์อาร์เอส ทิลมิโคซิน

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