

The inhibition of FMD virus excretion from the infected pigs by an antiviral agent, T-1105

Kenichi Sakamoto^{1*}, Seiichi Ohashi¹, Reiko Yamazoe¹, Kazumi Takahashi², and Yousuke Furuta²

¹ Exotic Diseases Research Station, National Institute of Animal Health, Kodaira, Tokyo 187-0022, Japan., ² Toyama Chemical Co., Ltd., 3-2-5 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

Abstract:

Pyrazinecarboxamide derivatives are known to have a potent anti-RNA viral activity. We evaluated their *in vitro* anti-FMDV activity and found out the efficacy and the possibility of one of the derivatives, T-1105 as a useful tool for prevention of spread of FMDV at the early stage of the infection in pigs.

The antiviral activity of T-1105 was 1.6µg/ml by means of 50% plaque reduction assay with FMDV O/JPN/2000. T-1105 was administrated by fed to four pigs at a dose of 200 mg/kg twice a day for 6 days starting just one hour before 10⁶TCID₅₀ of FMDV O/JPN/2000 inoculation. The other two pigs were only inoculated with same dose of the FMDV. The clinical signs and their antibodies against FMDV were checked. The serum, plasma, nasal swab samples were collected everyday for 8 days. Virus existence in plasma and nasal swab samples of all pigs were tested with virus plaque assay and the Real-time PCR.

In the plasma of the administrated pigs, T-1105 remained at high levels exceeding the antiviral concentrations. The pigs in non-administrated (control) group showed the typical clinical signs of FMD, but no clinical signs of FMD were observed in the administrated pigs. The virus was not detected from nasal swab samples of the administrated pig group by virus plaque assay and real-time PCR. In this group both antibodies titres of LPB ELISA and the virus neutralization test increased very little, comparing with those of the control group.

T-1105 is considered to be a viral RNA-dependent RNA polymerase inhibitor. By the oral administration of this compound, the FMDV inoculated pigs did not create any symptoms of the disease. And furthermore, at least the virus was not excreted from their nasal route. Since the viremia stage was not observed by real-time PCR with the plasma samples and the increase of the antibodies to FMDV was so slight that it was considered there was no virus replication in the pigs. It is suggested that this antiviral agent inhibits FMDV excretion from the virus inoculated pigs. It can be a powerful tool to control the disease in pigs, especially at the early stage of infection before the pigs produce antibodies against FMDV by infection and /or by vaccines.

Introduction:

The foot-and-mouth disease (FMD) is highly contagious and economically devastating disease of cloven-hoofed animals. The causative agent, foot-and-mouth disease virus (FMDV) is an aphtovirus of the picornavirus family and is serologically classified into seven distinct serotypes such as O, A, C, South African Territories (Sat) 1, Sat 2, Sat 3 and Asia 1. The diseases caused by those different serotypes are clinically indistinguishable.

The economic impact of FMD can be catastrophic when the outbreak occurs in the FMD free countries without vaccination in naïve animals, especially in pigs. The pigs play a role as an amplifier in the outbreaks because they excrete the virus a thousand times more than the other susceptible animals do (Donaldson & Ferris, 1980). There are such examples in the outbreaks in 1997, Taiwan (Dunn & Donaldson, 1997) and in 2001, UK (Gibbens et al. 2001).

To control the disease there are two policies of "test and slaughter" and/or "vaccination". The countries where the FMD outbreaks occur will decide to use either or both approaches, depending on the epidemiological situation of their outbreaks.

Several *in vitro* research works has been reported on the antiviral agents against FMDV (Kleina & Grubman 1992, Pariente et al. 2005, Gu et al. 2006). However there were no reports which had demonstrated the efficacy of these antiviral agent *in vivo* experiments. In this paper we will report that one of Pyrazinecarboxamide derivatives, T-1105 whose mechanism of action is considered to be the inhibition of RNA-dependent RNA polymerase activity (Furuta et al. 2005), has strong antiviral effect against FMDV both *in vitro* and *in vivo*. In addition, we will discuss that in the control of FMD the usage of the antiviral agent can be one of powerful tool to reduce the expansion of FMD outbreaks, which is different from the FMD control by vaccine approach.

Materials and Methods:

Cells and virus: The Japanese isolate, FMDV O/JPN/2000 was propagated four times in bovine kidney primary cells. The IBRS-2 cells were used for the virus titration, the plaque reduction assay and virus neutralization test.

Antiviral agents: Anti-HIV agent, Efavirenz, one of the Non-Nucleotide Reverse Transcriptase Inhibitors (NNRTI) was purchased. Pyrazinecarboxamide derivatives, T-705, T-1105 and T-1106 were kindly supplied by Toyama Chemical Co., LTD.

Pigs: Six pigs (LW breeding) were purchased and were subjected to in vivo studies.

Anti-FMDV activities of the compounds: In vitro detection of antiviral activity was carried out by means of 50% plaque reduction assays with monolayers of IBRS-2 cells in 6 wells microplates infected with about 30-50 plaque forming units of FMDV, O/JPN/2000 in 200 μ l of MEM at 37 $^{\circ}$ C for 1hr. The compounds were diluted in a series of 4-time concentration in the overlay of 1.5% methyl cellulose-MEM containing with 2% newborn calf serum and 10% tryptose phosphate broth. The 50% inhibition concentration (IC₅₀) of plaque formations were calculated by using the plaque counts of cultures with or without the compounds.

Inhibition test of FMDV excretion from the pigs: In vivo studies were performed by using pigs which were injected with 10⁶TCID₅₀ of FMDV O/JPN/2000 in a right side of fore-foot pad. The control group was only inoculated the same dose of the virus. The treated group that was administrated the most efficient anti-FMD compound, T-1105 and the control group consisted 4 pigs and 2 pigs respectively. One hour before the virus inoculation, 200mg/kg of T-1105 was administrated orally as mixed with their food and after the first administration the same dose of T-1105 was readministrated by the same route and the same manner twice a day for 6 days post the virus inoculation (dpi) (Fig. 1). The serum, plasma and nasal swab samples were collected everyday in 9 days observation period. The plasma and nasal swab samples were subjected to the virus titration assay by virus plaque assay and the real-time PCR to estimate the virus excretion and the viremia stage. The antibody titre was detected by the liquid phase blocking (LPB) ELISA and the virus neutralization test (VNT).

Results:

Anti-FMDV activity of the compounds: IC₅₀ of anti-HIV agent, Efavirenz, one of the NNRTI and Pyrazinecarboxamide derivatives such as T-705, T-1105 and T-1106 against FMDV O/JPN/2000 are shown in Table 1. It shows the antiviral activity of T-1105 (Fig. 2) was the strongest and its IC₅₀ was calculated to be 1.6 μ g/ml. This Antiviral activity was approximately 10 times higher than those of the other derivatives and 35-70 times higher than that of the anti-HIV agent, Efavirenz.

Clinical signs observed in the treated and control pigs: The control pigs showed typical clinical signs such as fever, vesicles on their feet and lameness. On the other hand, no clinical signs of FMD were observed in the treated pigs with T-1105 through the period of the animal experiment.

FMDV existence in blood: In order to detect the presence of the virus in blood, plasma samples were subjected to the real-time PCR. The Ct values of the plasma samples of the treated group are more than 40 for the first 6 dpi. However those values of the control pigs are 20 to 35 in 1 – 3 dpi (Fig. 3). In the blood samples of the administrated group the virus was not detected. The viremia stage was not observed in this group.

Serological test by means of LPB ELISA and VNT: The ELISA antibody titres of the control group increased from 4 dpi and reached to more than 1:360 after 5-6 dpi and the titres of VNT reached to 1:128 from 6 dpi (Fig.4). On the other hand, the titres of the ELISA of the treated group were no more than 1:45 and less than 1:32 by VNT (Fig. 5).

The virus excretion from the nasal route: The excretions of FMDV from nasal route were examined by means of both the virus plaque assay and the real-time PCR method. The virus was not detected from the nasal swab samples of the treated group by the plaque assay (Fig. 6). The Ct values of the samples were more than 40 except one of the first dpi samples (Ct = 36). The values of the control show 29 to 38 in 2-3 dpi nasal swab samples. The titres of the excretion viruses of the control pigs were 10² to 10⁴PFU /ml in 2 – 4 dpi of nasal swab samples (Fig. 6).

Discussion:

Pyrazinecarboxamide derivatives are known to have a potent anti-RNA viral activity. One of the derivatives, T-705 has antiviral activity against Poliovirus and Rhinovirus of Picornaviridae (Furuta et al. 2002). However, it was found out in our in vitro studies that T-1105 was more effective antiviral agent than T-705 against FMDV. The chemical structures of the T-1105 and T-705 are shown in Fig. 2. Their structural formula is the extremely same homologue. Since T-705 inhibits influenza virus RNA polymerase (Furuta et al. 2005), the mechanism of the action of T-1105 is considered to be the inhibition of FMDV RNA-dependent RNA polymerase.

As a pilot test it was demonstrated that by oral single administration of T-1105 at a dose of 100mg/kg the agent remained at high levels exceeding the antiviral concentrations in the plasma of the pigs for at least 15-20 hrs. T-1105 was considered not to be metabolized so easily and quickly in pigs.

The administrated pigs developed no clinical signs of FMD by the virus challenge and the FMDV excretion was not detected from their nasal route by either the plaque assay or the real-time PCR methods. Although only slight increase of the both ELISA and VN antibodies was observed, it is considered that inoculated FMDV is only recognized as foreign antigens but the viruses does not propagate in the pig body with the virus infection. These facts are suggesting that T-1105 is effective antiviral agent against FMD infection in pigs.

In general the pig excretes FMDV 100-2000 times more than the other susceptible animals do (Donaldson & Ferris, 1980). Furthermore, the virus is excreted at the early stage of the infection to the animals before they produce the antibodies to protect themselves from FMDV infection. At this early stage of infection the infected pigs shed a large amount of the viruses and they can be new origin of infections by airborne infection. To reduce this virus excretion at this early stage of FMDV infection, anti-FMDV agent which has an immediate effect on the inhibition of the virus propagation is needed.

In this paper we demonstrated that one of the Pyrazinecarboxamide derivatives, T-1105 had potent and selective inhibitory activity against FMDV in vivo. This anti-FMDV agent has advantages of an immediate effect at the early stage of the virus infection and complete inhibition of FMDV excretion from FMDV inoculated pigs. As this agent can be fed with their food, it can be administrated to a large number of the animal without a labour. The advantages compensate defects of the FMD vaccine such as the time lag until antibody is produced at protection level, vaccine matching with the field strain and a labour on vaccination. It is considered that the usage of this kind of excellent antiviral agents in FMD outbreaks of FMD free countries without vaccination can be a strong tool to control the disease and to reduce the expansion of FMD outbreaks. There is a possibility to reduce local spreads of FMD by such anti-FMD compound. From this point of view, it should be established that the usage of such anti-FMDV agents can be another new option in the control of FMD.

References:

- Donaldson, A.I. & ferris, N.P.** 1980. Sites of release of airborne foot-and-mouth disease virus from infected pigs. *Res. Vet. Sci.*, 29:315-319
- Dunn, C.S. & Donaldson, A.I.** 1997. Natural adaptation to pigs of a Taiwanese isolate of foot-and-mouth disease virus. *Vet. Rec.* 141:174-175
- Furuta, Y., Takahashi, K., Fukuda, Y., Kuno, M., Kamiyam, T., Kozaki, K., Nomura, N., Egawa, H., Mimani, S., Watanabe, Y., Narita, H., Shiraki, K.** 2002. In vitro and in viro activities of anti-Influenza virus compound T-705. *Antimicrob. Agents Chemother.* 46:977-981
- Furuta, Y., Takahashi, K., Kuno-Maekawa, M., Sangawa, H., Uehara, S., Kozaki, K., Nomura, N., Egawa, H., Shiraki, K.** 2005. Mechanism of Action of T-705 against influenza virus. *Antimicrob. Agents Chemother.* 49:981-986
- Gibbens, J.C., Sharpe, C.E., Wilesmith, J.W., Mansley, L.M., Michalopoulou, E., Ryan, J.B., Hudson, M.**, 2001. Descriptive epidemiology of the 2001 foot-and-mouth disease epidemic in Great Britain: the first five months. *Vet. Rec.* 149:729-743
- Gu, C.J., Zheng, C.Y., Shi, L.L., Li, Y., Qu, S.F.** 2006 An antiviral mechanism investigated with ribavirin as an RNA virus mutagen for foot-and-mouth disease virus. *J. Biochem. Mol. Biol.* 39:9-15
- Kleina, L.G. & Grubman, M.J.** 1992. Antiviral effects of a thiol protease inhibitor on foot-and-mouth disease virus. *J Virol.* 66: 7168-7175
- Pariente, N., Sierra, S., Airaksinen, A.** 2005. Action of mutagenic agents and antiviral inhibitors on foot-and-mouth disease virus. *Virus Res.* 107:183-193

Table 1 In vitro anti-FMD activities

Group of Compound	Agent	IC (µg/ml)
Anti-HIV agents (Non-Nucleotide Reverse Transcriptase Inhibitors)	Efavirenz	20 - 40
Pyrazinecarboxamide derivatives	T-705	14
	T-1105	1.6
	T-1106	17

Virus : FMDV O/JPN/2000 Cells : IBRS-2

Table 2 Clinical symptoms

Treatment	No.	Days post inoculation								
		0	1	2	3	4	5	6	7	8
T-1105	1	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-
Control	5	-	-	+	++	++	++	++	++	++
	6	-	-	-	+	+	++	++	++	++

+ Blister formation
 ++ Rupture

Lameness / Dysstasia

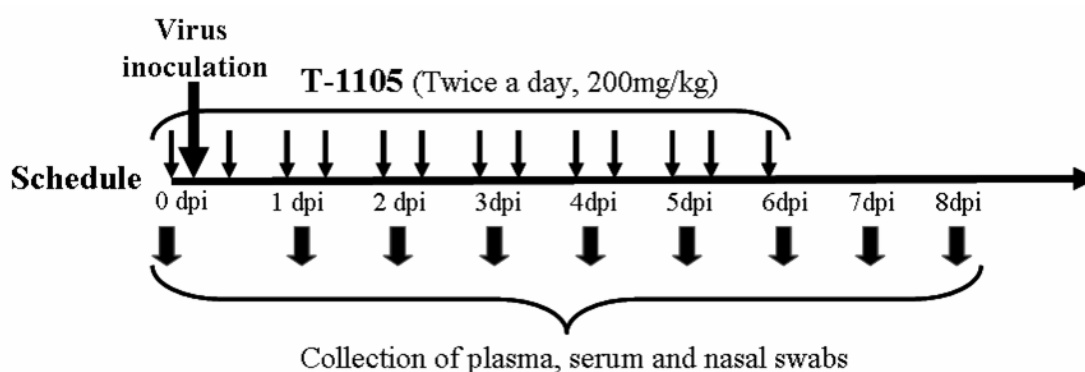


Fig.1 In vivo experiment.

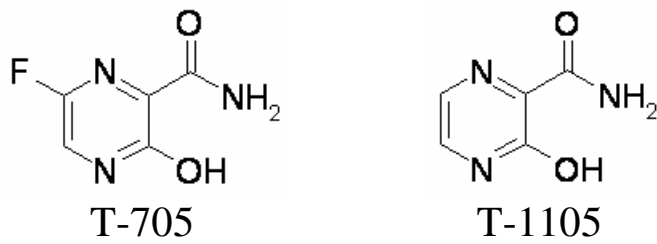


Fig.2 Structural formulas of T-1105 and T-705

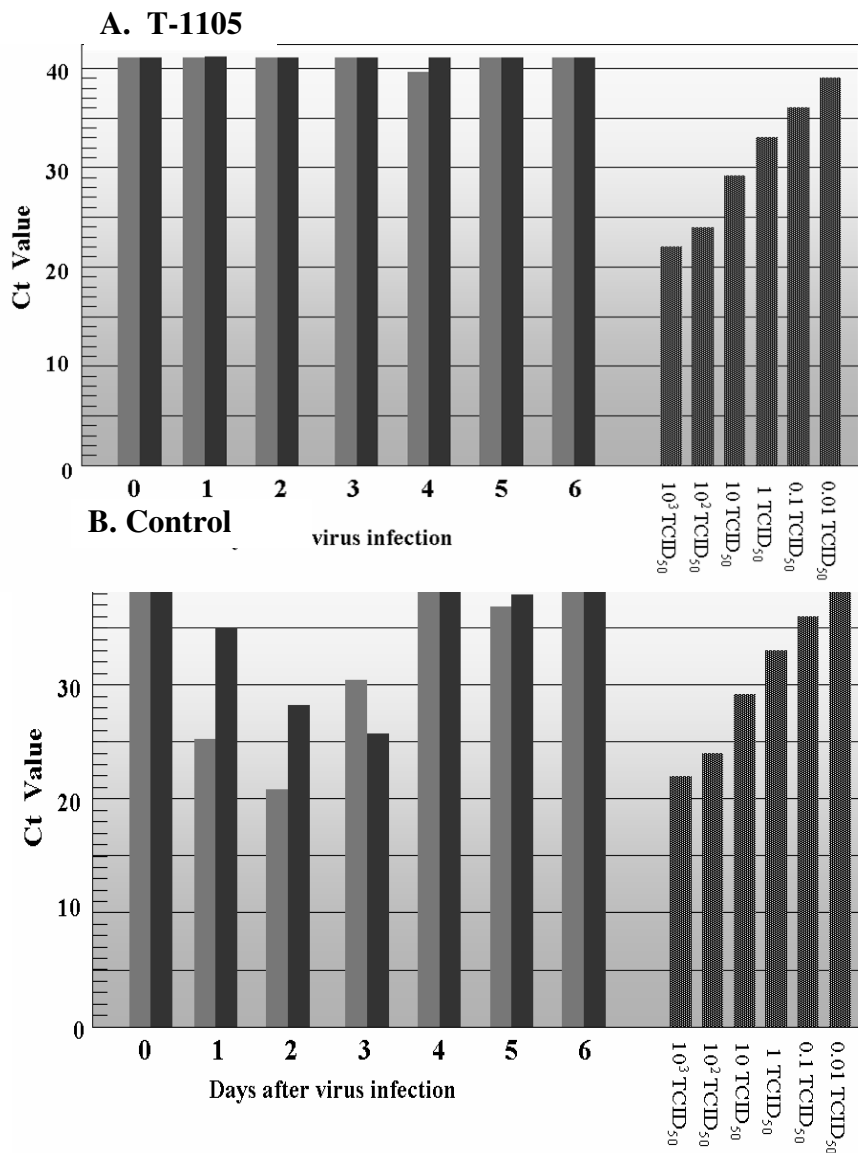


Fig.3 Real-time PCR result with plasma samples of the T-1105 administrated group (A) and the control group (B) FMDV was not detected through 0 – 6 dpi from all the plasma samples of the T-1105 administrated group. The Ct values were over 40 (A). The viruses were detected in 1 – 3 dpi plasma samples of the control pigs.

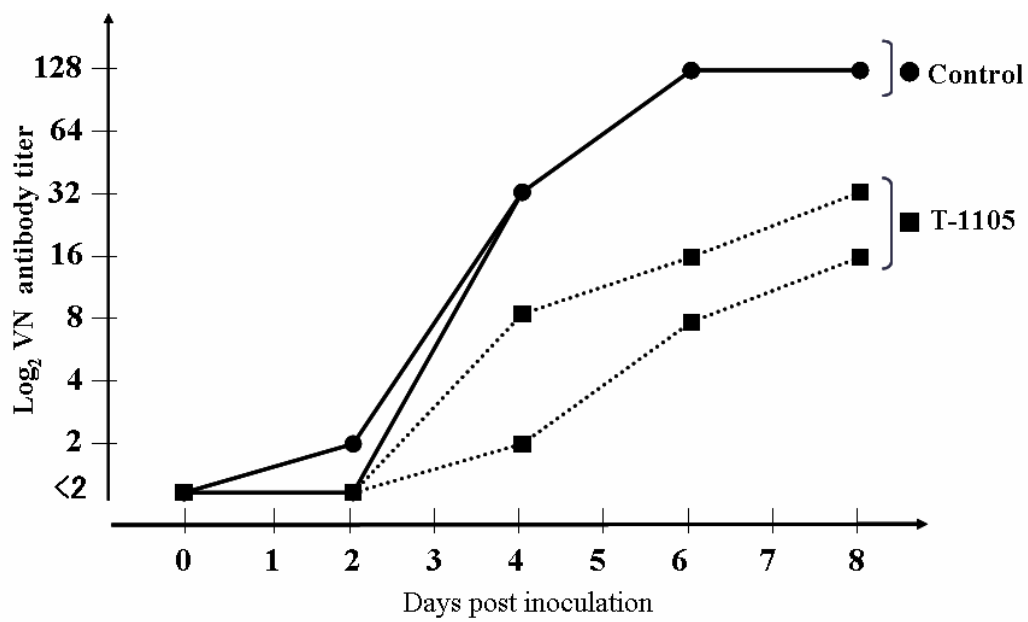


Fig.4 The titer of virus neutralization

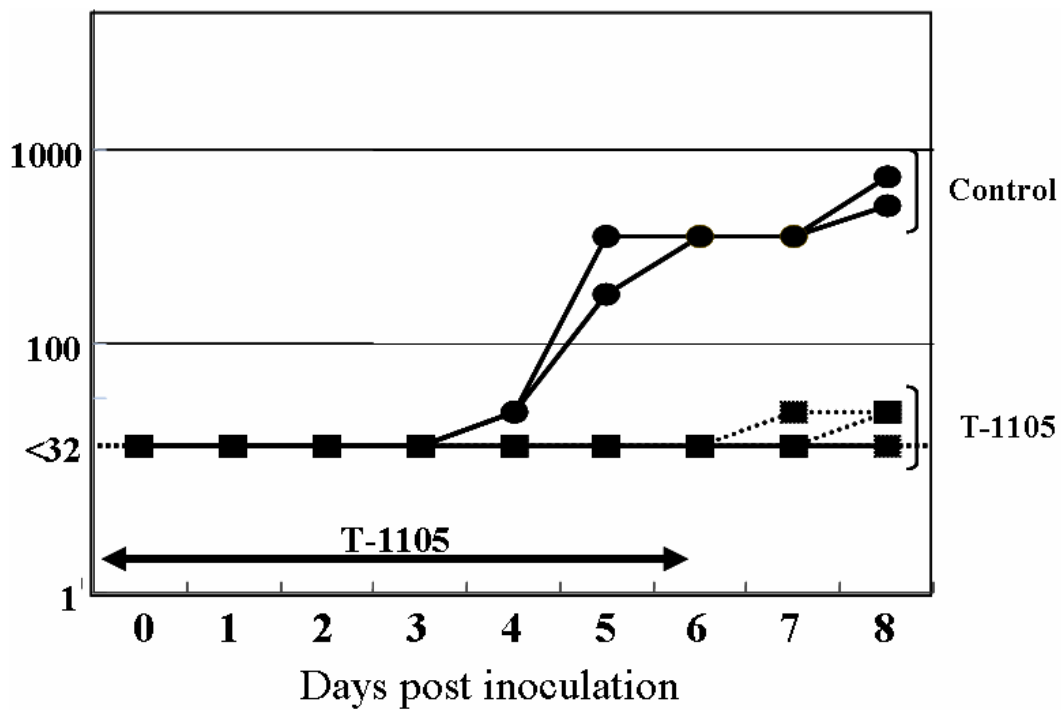


Fig.5 The titer of LPB ELISA

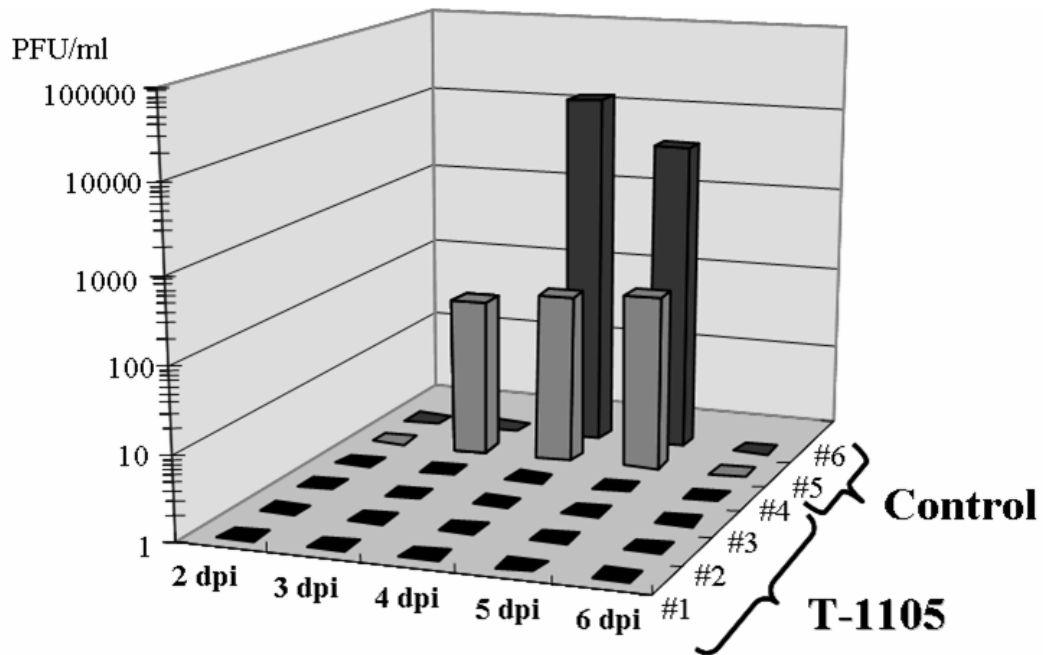


Fig.6 The virus was not detected from the nasal swab samples of the treated group by the plaque assay. The titres of the excretion viruses of the control pigs were 10^2 to 10^4 PFU /ml in 2 – 4 dpi of nasal swab samples.