

Replication of two porcine parvovirus isolates at non-permissive temperatures

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Summary. Previous studies have shown that replication in vitro of the porcine parvovirus (PPV) isolate, KBSH, was restricted at 39 °C but not at 37 °C. In contrast, replication of the Kresse isolate was restricted at 37 °C but not at 39 °C. In this study, Kresse and KBSH isolates were passaged up to ten times in swine testicle (ST) cells at non-permissive temperatures, and at subsequent passage viral protein synthesis, viral DNA synthesis, and progeny virus were evaluated. KBSH became adapted for replication at 39 °C upon serial passages, displaying an appreciable increase in viral progeny, viral polypeptides, and viral DNA concentration. This finding was also observed with Kresse virus isolate continuously passaged at 37 °C. Neither isolate became adapted for replication at 32 °C. In an attempt to examine the effect of in vitro passage at non-permissive temperatures on pathogenicity in swine, KBSH passaged 10 times either at 37 °C or 39 °C was inoculated into swine fetuses. Two of four fetuses inoculated with 39 °C-passaged KBSH were dead and hemorrhagic or mummified. All four fetuses inoculated with 39 °C-KBSH contained viral antigen and viral DNA. In contrast, fetuses inoculated with 37 °C-passaged KBSH, or with cell culture fluid were normal in appearance. Viral antigen and viral DNA were not demonstrated in fetuses inoculated with 37 °C-KBSH or cell culture fluids. These findings suggest the possibility that the ability to replicate at 39 °C is associated with virulence in swine fetuses.

Introduction

Porcine parvovirus (PPV), a member of the autonomous parvoviruses, causes reproductive failure in susceptible pregnant sows [14, 18]. A number of isolates of PPV have been made worldwide, some of which show obvious differences in pathogenicity [7, 16, 21].

In a previous study, four PPV isolates, NADL-8, NADL-2, KBSH, and Kresse, were compared for their replicative properties in vitro at either 32 °C,

37°C, or 39°C to explain the differences observed in the replication of these isolates in swine [5]. NADL-8 and NADL-2 are isolates pathogenic only to mid-term gestation fetuses [18, 23] when inoculated in utero. These two isolates showed similar replication patterns at 32°C, 37°C, and 39°C. However, replication of KBSH, an isolate which is not pathogenic to swine fetuses [21], was restricted at 39°C, but not at 32°C or 37°C. The replication of Kresse, an isolate which is pathogenic both to mid- and late-term fetuses as well as to young pigs [3, 16], was favored at 39°C and restricted at 32°C and 37°C.

Temperature sensitive (ts) mutants have been described for pseudorabies virus [2, 8], measles virus [6], coxsackie virus B3 [10], fowl plague virus [22], and parvoviruses, H-1 [26], Kilham rat virus [27], and PPV [9, 13]. H-1 parvovirus ts mutant synthesizes a nonsense capsid protein defective in hemagglutination and exhibits decreased synthesis of viral DNA, but not RF-DNA [26]. Ts mutants isolated from nitrous acid-treated KRV [27] show two distinct groups, one restricted in single stranded (SS) viral DNA and capsid protein production and the other restricted in accumulating RF-DNA. Low temperature adapted ts PPV mutants were shown to be effective when employed as modified live vaccines [9, 13]. Revertants of ts mutants have also been described and a possible mechanism for revertants has been suggested in various viruses [1, 15, 17, 24].

In order to understand the relationship between permissive temperatures for virus replication and their pathogenicity in vivo, virus isolates were serially passaged in vitro at non-permissive temperatures. Kresse and KBSH isolates were serially passaged at non-permissive temperatures, 37°C or 39°C. These passaged viruses were subsequently evaluated for their abilities to replicate in vitro in ST cells and in vivo following in utero exposure to swine fetuses. An important consideration in this study is that the mean body temperature of swine is 39.2°C in difference to 36.8°C for humans.

Materials and methods

Cells and viruses

An established cell line, swine testicle (ST) cells, was employed throughout this study for the propagation of PPV in vitro. Conditions for the culture of ST cells were followed as previously described [20]. The isolates of PPV chosen for this study included KBSH, obtained from Dr. P. Tattersall (Yale University, New Haven, CT) and Kresse from Dr. J. Kresse (NVSL, Ames, IA). KBSH, previously passaged approximately 300 times in KB cells at 37°C [11], had been propagated twice in ST cells at 37°C in our laboratory before use in this study. Kresse, originally isolated from skin lesions of young pigs [15], was propagated twice in ST cells and once in swine fetuses. Using these isolates, stock virus was prepared in ST cells at 37°C as previously described [5].

Continuous passages of isolates at non-permissive temperatures

Kresse and KBSH isolates were passaged in ST cells either at 32°C, 37°C, or 39°C and changes in virus progeny and virus antigen were evaluated. ST cells seeded at 1×10^6 cells per 25 cm² flask were infected at an m.o.i. of 1. Infected cells were harvested at 5 days

post-infection (PI) by 2 freezing and thawing cycles. For subsequent passages, the virus inoculum was adjusted by hemagglutinating units (HAU), 40 HAU per 75 cm² flask. At each passage level, infectious virus titers were determined by indirect immunofluorescence [19] and titers were expressed as fluorescent focus unit (FFU) [4]. In addition, viral protein and viral DNA synthesis were determined at selected passages (see below). Uninfected ST cells were passaged up to 10 times and served as controls.

PPV protein synthesis

Immunoprecipitation of ³⁵S-methionine radiolabelled polypeptides from infected cell lysates was performed to evaluate viral protein synthesis following procedures previously discussed [17] with one exception. ST cells were infected with an m.o.i. of 1 either at 32 °C, 37 °C, or 39 °C for 20 h. Cells were then pulsed for an additional 24 h with 150 µCi/ml of ³⁵S-methionine. The extended labelling times were necessary to observe the third structural protein of PPV, Vp3. Antisera used for immunoprecipitation was from rabbits hyperimmunized with CsCl purified PPV (1.39 g/ml fraction), NADL-8 isolate [19].

PPV DNA synthesis

To evaluate PPV DNA production, slot blot hybridization was performed on Hirt extracted DNA [25]. PPV isolates were propagated in ST cells either at 32 °C, 37 °C, or 39 °C, for 20 h, followed by DNA extraction. Extracted viral DNA was placed onto nylon membranes contained within a Manifold slot blot chamber (Schleicher and Schuell, Keene, NH). The membrane was hybridized with a PPV specific radiolabelled probe following procedures described previously [4].

Pathogenicities of serially passaged PPV isolates in swine fetuses

To evaluate the influence of temperature adaptation on pathogenicity, 37 °C and 39 °C passaged KBSH were inoculated into swine fetuses. Two sows, seropositive to PPV, at 57 days and 60 days of gestation, respectively, were subjected to laparotomies [21] to expose fetuses for in utero virus inoculation. Upon laparotomy it was learned that each sow had only 6 fetuses. Four fetuses from each sow were inoculated via the amniotic cavity with 10^{4.5} FFU/0.5 ml of KBSH passaged at either 37 °C or 39 °C. The remaining 3 fetuses were inoculated with uninfected cell culture extract, passaged 10 times at 39 °C without PPV infection. Fourteen days later, the sows were euthanized and the uteri were collected. Fetuses were observed for gross appearance and the age of death by measuring crown-rump (C-R) length. Tissues from each fetus were individually collected and homogenized to a 20% (w/v) suspension in TE buffer (50 mM Tris and 25 mM EDTA pH 8.7). Homogenized tissues were tested for HA antigen and viral DNA [12]. Relative densities in hybridizations were analyzed by scanning densitometer (Shimadzu Scientific Instrument, Columbia, MD) at a wavelength of 633 nm. Results were graphed comparing relative density to known concentrations of purified PPV RF-DNA, as determined by spectrophotometry.

Results

Continuous passages of PPV isolates at non-permissive temperatures

The production of infectious virus for both Kresse and KBSH either remained constant or increased at subsequent passage level, both at 37 °C and 39 °C, but not at 32 °C (Fig. 1). High infectious virus titers, 5 × 10⁶ to 8 × 10⁶ FFU/0.1 ml, were maintained at 39 °C through 10 passages for Kresse isolate replication (Fig. 1A). At 37 °C, infectious virus titers increased from 2 × 10³ FFU/0.1 ml at

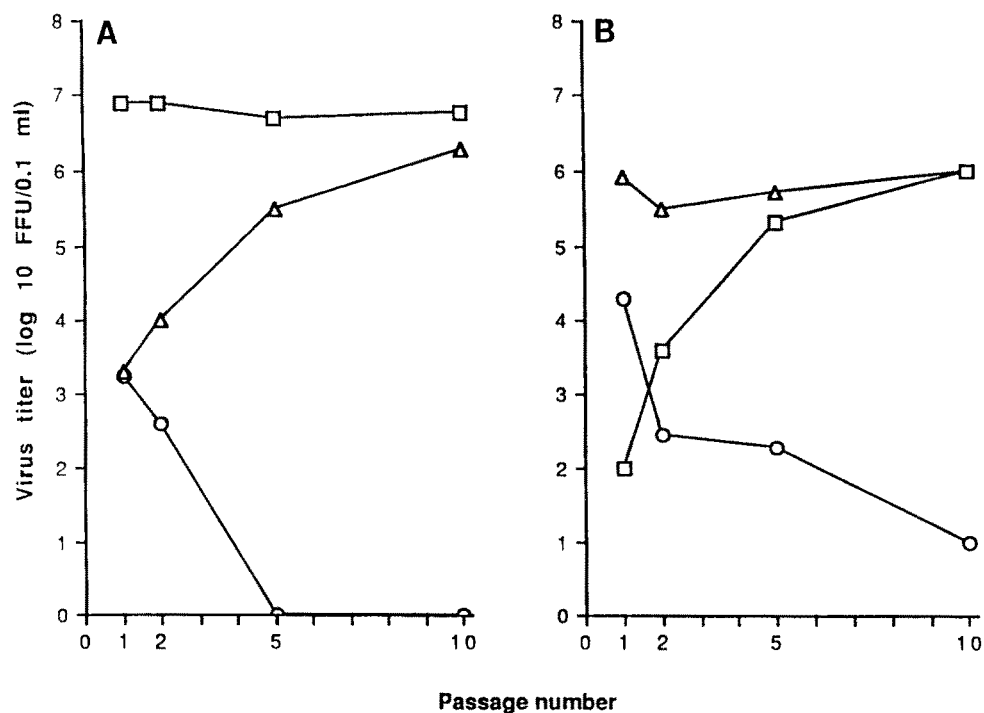


Fig. 1. Infectious virus titers of Kresse (A) and KBSH (B) virus isolates serially passaged at either 32°C (○---○), 37°C (△---△), or 39°C (□---□). At each passage level, cell-associated infectious virus was measured by indirect fluorescent antibody staining

the 1st passage to 2×10^6 FFU/0.1 ml at the 10th passage. At 32°C, virus titers decreased upon passages and infectious virus titers were not detected after the 5th passage. High titers of infectious virus, 3×10^5 FFU/0.1 ml to 1×10^6 FFU/0.1 ml, were maintained at 37°C for KBSH isolate replication (Fig. 1B). At 39°C, low titers of infectious virus titers, 1×10^2 FFU/0.1 ml, were found at the 1st passage which increased to 1×10^6 FFU/0.1 ml at the 10th passage. Again, at 32°C, infectious virus titers decreased from 2×10^4 FFU/0.1 ml at the 1st passage to 1×10^1 FFU/0.1 ml at the 10th passage. Infectious virus was not detected from uninfected ST cells passaged up to 10 times.

Viral polypeptides appeared as weaker bands at lower passage levels at non-permissive temperatures, 37°C for Kresse and 39°C for KBSH, compared to those at permissive temperatures. At subsequent passages, polypeptide bands of both isolates appeared more intense at non-permissive temperatures, 37°C for Kresse and 39°C for KBSH (Fig. 2B and C). This was especially true for Vp3 which was more prominent in passages 5 and 10 indicating more complete full virus particle production. Furthermore, bands corresponding to polypeptide Vp3 were more intense at 39°C than at 37°C for both isolates. Viral polypeptides were undetectable when viruses were passaged at 32°C, even with longer exposure times (Fig. 2A).

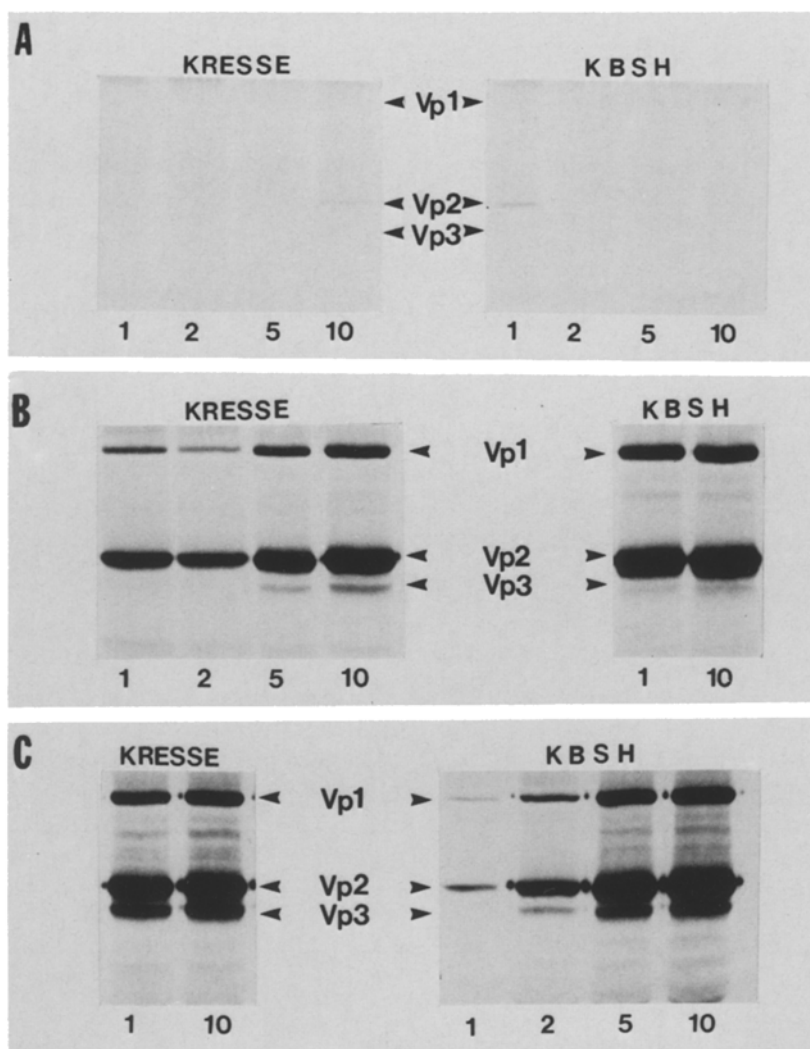


Fig. 2. Viral protein synthesis of Kresse and KBSH upon passage at either 32 °C (A), 37 °C (B), or 39 °C (C). Radiolabelled ^{35}S -polypeptides were immunoprecipitated with rabbit anti-PPV sera, electrophoresed, and exposed to X-ray film. Numbers on the left hand margin represent molecular weight markers

Similar findings to polypeptide synthesis were observed for viral DNA synthesis (Fig. 3). Serial passage at 37 °C for Kresse and at 39 °C for KBSH increased hybridization signals.

Pathogenicity of serial passaged PPV isolates

Previous studies have shown Kresse isolate to replicate and cause fetal death in midgestation and late gestation swine fetuses [3]. These original experiments in swine fetuses were undertaken with a virus isolate from clinical samples passaged once in swine fetuses to prepare stock virus. Such passaged virus

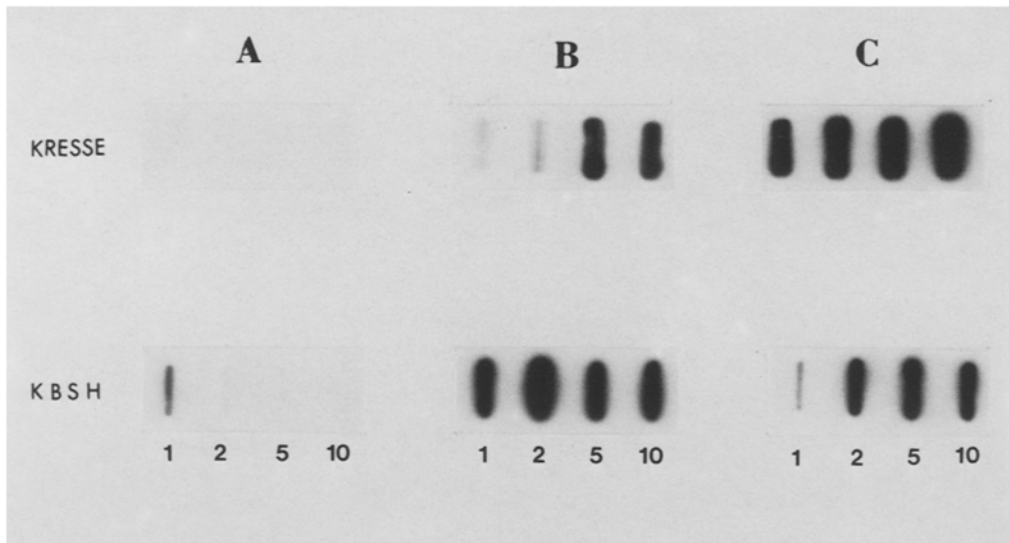


Fig. 3. Viral DNA synthesis of Kresse and KBSH isolates upon serial passage at 32 °C (A), 37 °C (B), and 39 °C (C). Extracted DNA was transferred onto nylon membranes and hybridized with a PPV specific ³²P-labelled probe followed by exposure to X-ray film

Table 1. Pathogenicity of KBSH virus, passaged in vitro at either 37 °C or 39 °C, in swine fetuses

Inoculum	No. fetus ^a	Gross appearance ^b (no. fetus)			Virus detection (no. fetus)	Antibody response (no. fetus)
		N	H	M		
KBSH-37 °C	4	4	0	0	0	0
KBSH-39 °C	4	2	1	1	4	3
Control	4	4	0	0	0	0

^a Two fetuses from each of two sows were inoculated with either KBSH-37 °C (10 times passage), KBSH-39 °C (10 times passage) or cell culture media (10 times passage)

^b The gross physical condition of fetuses was noted as normal (*N*), hemorrhagic (*H*), or mummified (*M*)

remained highly pathogenic to swine fetuses. In contrast, previous experiments with KBSH [21] had failed to demonstrate evidence of virus replication in swine fetuses. The KBSH virus stock had been passaged at least 300 times in KB cells [11] and ST cells [21] all at 37 °C before in vivo experiments. In this experiment we focused on whether serial passage of KBSH virus affected its ability to replicate in swine viruses. While all fetuses inoculated with 37 °C-passaged KBSH isolate were normal in appearance, two fetuses (one from each sow) inoculated with 39 °C-passaged KBSH isolate were abnormal; one was slightly hemorrhagic, and another was mummified (Table 1). To ascertain the

Table 2. Virus spread in tissues of swine fetuses inoculated with KBSH in vitro passaged at either 37 °C or 39 °C

Inoculum	Fetus	Virus detection (HA ^a /DNA ^b)				
		spleen	kidney	liver	lung	brain
KBSH-37 °C	1	-/-	-/-	-/-	-/-	-/-
	2	-/-	-/-	-/-	-/-	-/-
	3	-/-	-/-	-/-	-/-	-/-
	4	-/-	-/-	-/-	-/-	-/-
KBSH-39 °C	1	NT/NT	32/++	1,024/+++	32/+	256/++
	2	128/++	-/-	4,096/+++	-/+	-/+
	3	-/+	-/-	-/+	-/-	-/-
	4	16/++	-/-	-/-	-/-	-/-
Control ^c	1	-/-	-/-	-/-	-/-	-/-
	2	-/-	-/-	-/-	-/-	-/-
	3	-/-	-/-	-/-	-/-	-/-
	4	-/-	-/-	-/-	-/-	-/-

^a HA titers less than 1:2 were recorded as negative (-)

^b Viral DNA was detected by slot blot hybridization, and results were recorded from - to +++ by intensity. +++, ++ and + were approximately equivalent to 10 ng, 1 ng and 0.1 ng per 10 µl of RF-DNA, respectively

^c Supernatant of ST cell cultures passaged 10 times at 39 °C were inoculated as control

association of PPV with fetal death, tissues of each fetuses were evaluated for the presence of viral antigen and viral DNA (Table 2). All four fetuses inoculated with 39 °C-passaged KBSH isolate exhibited viral antigen and viral DNA in various tissues, such as spleen, kidney, liver, lung, and brain. However, no HA antigen or viral DNA was demonstrated from tissues of fetuses inoculated with 37 °C-passaged KBSH or cell culture fluid.

Discussion

Kresse and KBSH isolates were serially passaged at non-permissive temperatures in an attempt to evaluate the effect of in vitro passages on the pattern of virus replication. This study was prompted by previous work that showed markedly different replication patterns in Kresse and KBSH isolates at 32 °C, 37 °C, and 39 °C [5].

Multiple passages of Kresse isolate at 37 °C and KBSH at 39 °C yielded higher titers of infectious virus compared to those of non-passaged isolates. However, at 32 °C, low titers of infectious virus were produced up to the 10th passage with Kresse isolate, and decreased infectious virus titer was observed with the KBSH isolate. These findings indicate that two isolates of PPV passaged 10 times became adapted for growth at 37 °C or 39 °C. Neither isolate became

adapted to growth at 32 °C, following 10 passages. This finding may be due to the slow doubling times of ST cells at 32 °C rather than an inability to replicate at such temperature.

A possible selection mechanism is that in mixed phenotypes of a virus preparation, one phenotype becomes dependent on temperature. Previous studies indicated that restricted replication at a non-permissive temperature may be due to a defect in virus assembly for KBSH, but replication of Kresse appeared to be compromised at the initial stage of replication [5]. Since KBSH had been passaged more than 300 times in KB cells [11] and ST cells at 37 °C [21], and Kresse had been passaged only 3 times in ST cells at 37 °C, a possible explanation for temperature dependent replication of each isolate is that one phenotype which can grow at 37 °C for KBSH or at 39 °C for Kresse became dominant in the virus preparation. It is possible that these virus preparations consist of two populations of mixed phenotypes. By passage at a non-permissive temperature, phenotypes which can grow at non-permissive temperatures might be generated and became dominant by natural selection. It is unlikely that reversion is caused by mutation because reversion occurred at low passages (less than 10 times). In a previous report [21], KBSH was not pathogenic to swine fetuses when inoculated in utero with KBSH. However, it could not be excluded that KBSH upon blind passage in swine fetuses may cause fetal death. The role of DI particles in selection mechanism for reversion remains unknown due to a paucity of information on DI particles of PPV.

Analyses of viral polypeptides in this study, at either 37 °C or 39 °C, demonstrated that polypeptide synthesis of both isolates increased by serial passages at non-permissive temperatures. This was especially true with Vp3 suggesting that more full virus particles were produced. Alternatively, more effective protein cleavage may occur at 39 °C. Although polypeptides corresponding to Vp3 were more intense at 39 °C than at 37 °C for both Kresse and KBSH, progeny virus titers were comparable. In PPV, the ratio of empty to full virus particles in cell cultures are often greater than 50:1 in cell culture systems compared to 1:1 following experimental infection in animals [4]. Previous studies showed that inhibition of PPV replication by empty particles were dependent upon the concentration of empty particles present in virus preparation in cell cultures and in animals [4]. Comparable progeny virus titers, despite differences in the concentration of Vp3, may be due to higher concentrations of empty particles produced in proportion to the production of more full virus particles.

KBSH appeared to gain the ability to replicate in swine fetuses following serial cell culture passages at 39 °C evidenced by fetal death and virus detection in fetal tissues. This result can be supported by previous reports that the replication at near the body temperatures were correlated with virulence in polio-virus [17] and mouse corona virus [15]. Compared to experimental fetal infection with NADL-8 or Kresse isolate [3, 16], fetal abnormalities were less severe. One of the explanations for this may be the age of fetuses. Joo et al. [14] suggested that the most susceptible age to PPV infection was between 32

and 56 days of gestational age. The age of swine fetuses used in this study was between 55 and 60 days. It should be noted that in difference to NADL-8, Kresse isolate is highly pathogenic in late term swine fetuses [3]. Another possibility is that there was insufficient adaptation following 10 passages to induce fetal death in late gestational age swine. Further experiments with virus passaged greater than 10 times and fetuses at various gestational ages will address this question. KBSH stock virus used in this study was not end-point cloned. Because a markedly different growth profile was observed between KBSH and Kresse [5], this study was focussed on the pathogenic changes of these isolates upon serial passages at nonpermissible temperatures. It may be valuable to characterize the mixed clones by end-point cloning for further study.

In conclusion, KBSH and Kresse isolates became adapted to 39°C or 37°C, respectively, by serial passages. The adaptation was evidenced by increased viral antigen, viral polypeptides, especially Vp3, viral DNA, and infectious virus. Furthermore, KBSH isolate passaged at 39°C for 10 times now showed the ability to replicate in swine fetuses, indicating that pathogenicity can be recovered by serial adaptation at the mean body temperature of pigs, 39°C.

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