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A novel single-cell quantitative real-time RT-PCR method for quantifying foot-and-mouth disease viral RNA

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Foot-and-mouth disease virus is a positive-sense, single-stranded RNA virus with a negative strand as its replication intermediate, which can cause severe acute infection in sensitive cell lines. To investigate better the actual state of virus infection, there is a need to measure the amount of FMDV RNA in a single acutely infected cell rather than in a large number of cells. Therefore, in the present study, a strand-specific single-cell quantitative real-time RT-PCR was developed to analyze the RNA or FMDV. This new method uses two techniques in concert with each other: a technique for isolating single cells with micromanipulators, which is coupled to an assay for detecting viral RNA by real-time RT-PCR. In the assay of acute infection, 185 of 224 (82.6%) single-cell samples were positive and contained viral genome copies ranging from several to thousands, and up to 1 000 000 copies. However, not all cells were infected and there were differences in the number of viral RNA copies between cells. A single-cell quantitative RT-PCR was validated to be feasible and effective.

1. Introduction

Foot-and-mouth disease virus (FMDV) is the etiological agent of an important disease of cloven-hoofed livestock and wild animals, including cattle, swine, sheep and goats (Alexandersen and Mowat, 2005; Alexandersen et al., 2003). FMDV belongs to the *Aphthovirus* genus of the *Picornaviridae* family; the virus contains only one single-stranded, plus-sense RNA genome of approximately 8500 nucleotides surrounded by four structural proteins to form an icosahedral capsid (Belsham, 1993). The virus has seven different serotypes: A, O, C, Asia1, and South African Territories 1 (SAT 1), SAT2, SAT3 with a large number of subtypes (Grubman and Baxt, 2004).

FMDV, like other RNA viruses, has very high mutation rates, ranging 10^{-3} to 10^{-5} per nucleotide site per genome replication, owing to the lack of error correction mechanisms during RNA replication (Domingo et al., 2003). The FMDV genome RNA replicates via a complementary negative-strand RNA, which is used as a template (Cleaves et al., 1981). When sensitive cell lines are infected (e.g. BHK-21 cell lines), i.e., acute infection, cytopathic effects (CPE) appear, which involve cell rounding, and alteration and redistribution of internal cellular membranes. However, FMDV can also establish persistent infection not only in live animals but also in cell cultures. Carrier cells can be obtained by either using the ammonium chloride method (Gu et al., 2003) to select rapidly target cells or by incubating the cells surviving the cytolytic infection (De la Torre et al., 1985).

On the basis of the characteristics of FMDV genome, a number of methods for detection have been established, including hybridization assays (Verheyden et al., 2003), conventional RT-PCR (Moss and Hass, 1999; Hofmann et al., 2000) and real-time RT-PCR (Callahan et al., 2002; King et al., 2006; Gu et al., 2007; Horsington and Zhang, 2007; Shaw et al., 2007; Fosgate et al., 2008). Undoubtedly, real-time RT-PCR has advantages compared with the other techniques, including greater sensitivity, reproducibility, reduced risk of carry-over contamination, and greater rapidity, as results can be obtained in about 2 h. To date, all of these techniques are limited in that they require a large number of cells either within a certain tissue or a specific cell line; thus, only generalized data were obtained instead of precise and specific information at the level of the individual cell. Nevertheless, techniques for research on single cells to detect and quantify the viral RNA copies have been evaluated.

In the late 1980s, the feasibility of single-cell PCR as well as single-cell RT-PCR was explored. Jeffreys et al. (1988) amplified hypervariable minisatellites from single human cells using PCR. Mattano et al. (1992) and Krapf and Solioz (1991) performed RT-PCR

Abbreviations: BHK-21 cells, baby hamster kidney-21 cells; CPE, cytopathic effect; C_T, cycle threshold; FBS, fetal bovine serum; FMDV, foot-and-mouth disease virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h.p.i., hours post-infection; MEM, minimum essential medium; RT-PCR, reverse transcriptase polymerase chain reaction; qRT-PCR, quantitative real-time RT-PCR; sc-qRT-PCR, single-cell quantitative real-time RT-PCR.

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on single cells. After the emergence of quantitative real-time RT-PCR, the technique was applied successfully to quantify the number of mRNA copies in single neurons (Alsbo et al., 2001; Parhar et al., 2003; Hillman et al., 2005; Wagatsuma et al., 2005), in single cells during embryonic development (Rice et al., 2002; Pierce et al., 2002, 2003; Hartshorn et al., 2005, 2007) and in individual muscle fibers (Jemiolo and Trappe, 2004; Wacker et al., 2008). The present study combines real-time RT-PCR with single-cell techniques for detecting FMDV genomic RNA copies in single cells during acute infection.

2. Materials and methods

2.1. Cells and viruses

The virus strains of serotype O FMDV used in the present study were obtained from the Lanzhou Veterinary Research Institute, Chinese Academy of Agriculture Sciences. BHK-21 cells were provided by the China Center for Type Culture Collection (CCTCC). Cells were cultured in Minimum Essential Medium (MEM, GiBCO, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GiBCO, U.S.A.) at 37 °C with 5% CO_2.

2.2. Isolation and preparation of single cells

2.2.1. Isolation of single cells

Acutely infected cells, with CPE, were rinsed with PBS three times and then trypsinized at 37 °C for 5 min; 9 ml 10% FBS MEM was then added to end trypsinization. 1 ml of the mixed liquor was transferred to a dish at a density of 10^5 cells per dish. Cells were visualized using a phase-contrast microscope (Olympus, Japan) and isolated using a micromanipulator (Narashige, Japan) fitted with a microcapillary attached to a microinjector (Narashige, Japan) (Ponnaiya et al., 2007). Single cells were put into 0.2 ml DEPC-treated PCR tubes containing 5 μl 0.9% NaCl solution and 10 U RNase inhibitor (TAKARA, China) kept at room temperature (Liu et al., 2004). Approximately 30 individual cells can be picked and placed in individual tubes in 30 min. Every 30 min, the single-cell samples were frozen rapidly in liquid nitrogen for more than 2 min.

2.2.2. Lysis of single-cell samples

Frozen cells were heat-denatured at 98 °C for 3 min to break the cell membranes and were then immensed in liquid nitrogen as quickly as possible. The frozen samples were then digested with 1 μg proteinase K and 10 U RNase inhibitor at 53 °C for 1 h (Parhar et al., 2003). This was followed immediately by single-cell RT-PCR and real-time RT-PCR.

2.3. Virus infectivity assay (co-cultivation)

Monolayer BHK-21 cells were infected with FMDV (100 plaque forming units; PFU) and incubated in MEM supplemented with 2% FBS. When CPE appeared, single cells were isolated and put onto a 96-well plate (one cell per well) containing confluent normal cells. Morphologic alterations were observed under a microscope.

2.4. Single-cell RT-PCR

As not all cells were infected (Kullberg et al., 2006), GAPDH mRNA was determined as a positive control to prevent the emergence of false negative results and guarantee isolation of single cells. Specific primers for GAPDH (GenBank accession no. DQ403055) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) with the forward primer 5′-TGGCAAGTTCAAAGGCACA-3′ and the reverse primer 5′-AGATCCACGACGGACACG-3′, amplifying a 576 bp fragment.

Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega, U.S.A.) at a final volume of 25 μl according to the protocol. 1 μl of the treated single-cell sample was added to the mixture for a final concentration of 1 μM RT primer, 500 μM of each dNTP, 40 U RNase inhibitor (TAKARA, China), 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 200 U M-MLV Reverse Transcriptase. The mixture was vortexed and briefly centrifuged, then placed at 42 °C for 50 min, followed by 98 °C for 30 min to inactivate reverse transcriptase.

PCR reactions were performed in a final volume of 50 μl using Premix Taq (TAKARA, China), including 1.25 U Taq DNA polymerase, 400 μM of each dNTP, 4 mM Mg^{2+}, 0.5 μM of both the forward primer and reverse primer, 2 μl RT products. The reaction comprised 2 min of initial denaturation at 95 °C followed by 45 cycles consisting of 95 °C for 40 s, 55 °C for 30 s and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR products were then cooled and displayed on an ethidium bromide-stained 1.5% agarose gel. Gels were imaged using a bio-imaging system (Gene Genius, U.S.A.).

2.5. One-step single-cell real-time RT-PCR assay

RT-PCR was performed using the Platinum® Quantitative RT-PCR ThermoScript™ one-step Mastermix Reagents Kit (Invitrogen, U.S.A.), which combines cDNA synthesis and PCR in a single tube without additional handling between the two reactions. The PCR primers and probe for detecting FMDV RNA were located within the viral 3D genes encoding RNA-dependent RNA polymerase, which shows the least variation between serotypes, which is based on nucleotide sequencing of serotype O (Akesu/58/2002, GenBank accession no. AF511039; Gu et al., 2007). Primers and probes were designed using Primer Express 2.0; the primers were 5′-GAACACATTCTTTACACCAGGAT-3′ and 5′-CATATCTTTGCCAATCAACATCAG-3′, and the probe was 5′-FAM-ACAACCTACCGCCGAGCCAATTC-TAMRA-3′, amplifying a 120 bp fragment.

The standard RNA templates were prepared as follows. First, fragments within a 3D region were reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, U.S.A.) and amplified by PCR using pfu ultra™ High-Fidelity DNA polymerase (Stratagene, U.S.A.). The RT-PCR products were subjected to agarose gel electrophoresis to identify the target band, which was excised from the gel and DNA was isolated and purified using a Gel Extraction Kit (Omega, U.S.A.) according to the manufacturer's instructions. Second, this purified fragment was ligated into a pGEM-T plasmid vector (Promega, U.S.A.) and cloned to *E. coli* DH5α cells, and the recombinant was sequenced to confirm the orientation. Finally, after linearizing with the NcoI, target fragments were employed as templates for *in vitro* transcription using SP6 RNA polymerase (TAKARA, China) and the transcripts were digested with Rnase-free DNase I (Promega, U.S.A.), prior to purification with the RNeasy Mini Kit (Qiagen, Germany). The concentration of purified RNA transcripts was detected by a Bio Photometer (Eppendorf, Germany) at 260 nm. The RNA transcripts were aliquoted and stored at −80 °C until use.

The RT-PCR reaction was performed in a final volume of 50 μl consisting of 3 mM $MgSO_4$, 0.2 mM of each dNTP, 1 μl ThermoScript™ Plus/Platinum® *Taq* Enzyme Mix, 40 U RNase inhibitor, 300 nM Fluorogenic probe, 500 nM forward primer and reverse primer, 5 μg bovine serum albumin (New England BioLabs, U.S.A.) and 10 μl RNA sample or standard RNA samples. In addition, one drop of mineral oil was added to each tube to prevent vaporizing. The RT-PCR reaction was carried out on Rotor-Gene 2000

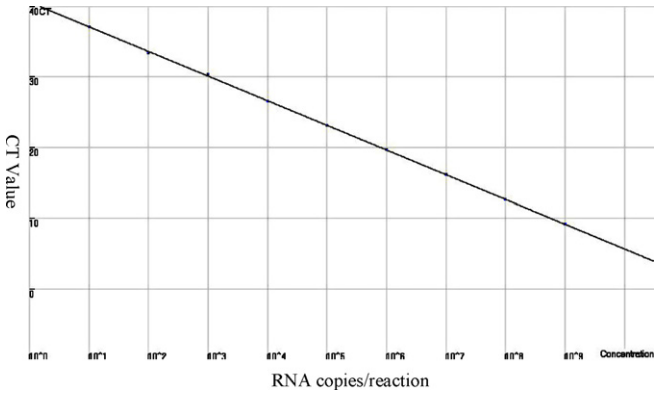

Fig. 1. Quantitation curve of FMDV RNA amplification. A 10-fold serial dilution of plus-stranded RNA transcribed in vitro was used as standard templates to produce a standard curve on the Rotor Gene 2000 real-time thermal cycler. The lower detection level was 10 copies per reaction. As given by the Rotor-Gene 4.6 software: slope = −3.497; R = 0.9999.

Real-Time thermal cycle (Corbett, Australia). Based on the manufacturer's protocol, cDNA was synthesized at 50 °C for 30 min, and the PCR profile was 95 °C for 5 min, followed by 45 cycles of 94 °C for 30 s and 60 °C for 90 s. Data analyses were carried out using Rotor-Gene 4.6.

3. Results

3.1. Specificity and quantitation limits of the real-time RT-PCR assay

The primers and Taqman probe were designed based on the FMDV 3D region, which is the most conserved and shows the greatest difference compared with the host cell genomes. The specificity of the assay was evaluated previously (Gu et al., 2007). After the real-time RT-PCR assay, the products were run on a 2% agarose gel and only a band between 100 and 250 bp was visualized.

The detection and quantitation limits were determined using C_T values obtained for ten serial dilutions ranging from 10^9 to 10^1 copies of the standard RNA. The values were plotted against the $\log_{10}$ of the number of template copies and a linear equation (copy number = $10^{(-0.286*CT+11.629)}$) was generated with a R^2 value = 0.9999 (Fig. 1). The assay maintained linearity for at least nine orders of magnitude. Using the slope from the linear equation, the overall efficiency of the assay was estimated to be 93%. The assay was negative below 10 template copes and, based on these results, samples with C_T value ≥37 were considered to be negative.

Table 1
Reproducibility of the qRT-PCR assay.

Sample number	Average C_T[a]	S.E.[b]
1	19.29	±0.048
2	25.97	±0.052
3	30.14	±0.032

[a] Average C_T value from three independent assay runs.
[b] Standard error of the average C_T values.

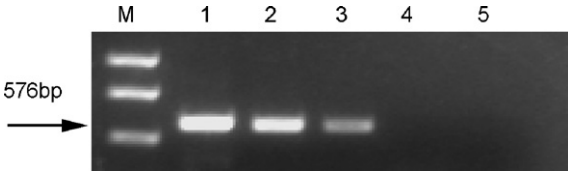

Fig. 3. Detection limits of the house keeping gene GAPDH mRNA in single-cell samples. M, molecular weight marker. Lanes 1–4 represent different dilutions of a single-cell sample. Lane 1: 1/5th of the single-cell sample; lane 2: 1/50th of the single-cell sample; lane 3: 1/500th of the single-cell sample; lane 4: 1/5000th of the single-cell sample; lane 5: negative control.

3.2. Reproducibility of the assay

Three different samples were tested in triplicate to verify the reproducibility of the assay. For each group, the three amplification curves overlapped with the standard error of the average C_T values of less than 0.1, which confirms the reproducibility of the assay (Fig. 2 and Table 1).

3.3. Isolation and lysis of single-cell samples

3.3.1. Single-cell RT-PCR of GAPDH

The detection limits of this RT-PCR were carried out using serial dilutions of single-cell samples (from 2×10^{-1} to 2×10^{-4}). In the experiment the threshold for detection was 0.2% of the content of one cell (Fig. 3). Therefore, as described in Section 2.4, one-fifth of a cell was used as template, which was sufficient for analysis. To confirm the accuracy of isolating single cells, this assay was undertaken on real-time RT-PCR with single-cell samples (Fig. 4 shows one of

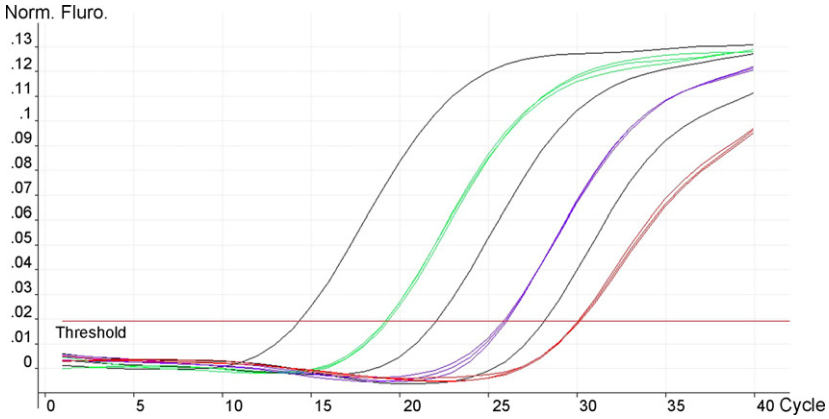

Fig. 2. Amplification curve of FMDV genome RNA. Three different samples (marked with green, violet and red curves) underwent qRT-PCR. The amplification curve in each sample overlapped perfectly and confirmed reproducibility of the assay. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

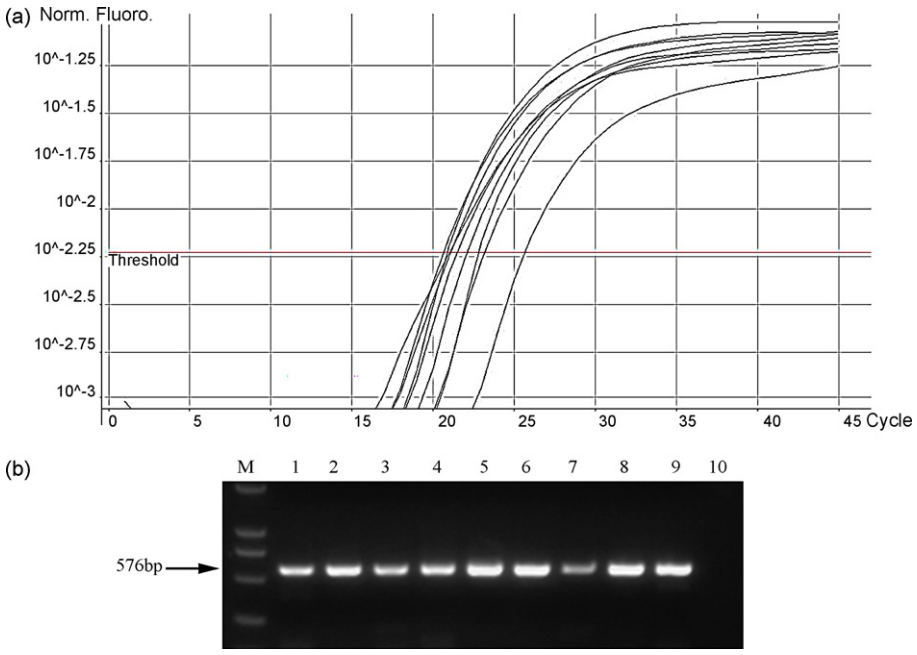

Fig. 4. Representative RT-PCR assay of GAPDH mRNA in single-cell samples. (A) Amplification curve obtained by sc-qRT-PCR. (B) Corresponding RT-PCR assay results of nine single-cell samples.

the assays). All single-cell samples tested at random were positive results, which demonstrated that the process of cell isolation was reliable and reproducible for real-time RT-PCR.

3.3.2. *Lysis of single-cell samples*

Standard RNA templates were introduced as control RNAs to evaluate RNA degradation during cell lysis. 5 μl control RNA (1000 copies of standard RNA) was added to 0.9% NaCl containing one normal BHK-21 cell and 20 U of RNase inhibitor. This was followed by heat denaturation and protease lysis. The solution containing both the single-cell and control RNA underwent real-time RT-PCR (Table 2). The average C_T value (27.62) of the treated samples was almost equal to or only slightly lower than that of the untreated samples (27.61). Consequently, this result indicated that the cell lysis process used in the present study (including heat denaturation and protease lysis) did not lead to RNA degradation.

Table 2
The stability of RNA in lysis of cell samples.

	C_T value	RNA copies
Control[a]	27.61	1042
Sample 1[b]	27.54	1089
Sample 2	27.66	1004
Sample 3	27.65	1009
Sample 4	27.63	1025

[a] Control RNA refers to untreated standard RNA. The C_T value and copy number were the average of three replicates.
[b] Samples 1–4 represent standard RNAs, which underwent cell lysis.

3.4. *Virus infectivity assay*

After the development of CPE, single cells were isolated for co-culture with normal BHK-21 cells laid on a 96-well plate. This assay was used to determine the percentage of cells possessing infectivity in a certain population (cells growing in T-25 flask), which were inoculated with viruses by the single-cell isolation method. At 18, 24 and 30 h post-infection (h.p.i.), infected cells were isolated and added to the wells of a 96-well plate, which was observed every 8 h to monitor alterations in cell appearance. Three different sampling time-courses were tested in triplicate; results are shown in Table 3 and Fig. 5. As shown in Table 2, not all of the infected cells contained complete virions, even although most cells exhibited cytopathic

Table 3
Infectivity of single cells inoculated with FMDV.

Hours post-infection[a]	The number of cells isolated	The number of cells appearing CPE[b]	Positive ratio (%)[c]	Average ratio (%)
18	96	46	47.9	48.9
	90	45	50	
	92	45	48.9	
24	88	68	77.3	76.3
	96	86	75.9	
	90	68	75.6	
30	92	68	73.9	73.0
	85	61	71.8	
	94	69	73.4	

[a] The post-infection morphological features are shown in Fig. 3.
[b] The number of cells with CPE was calculated within 120 h.
[c] The positive ratio equals the number of cells with CPE dividing by the total number of cells isolated. Once the cells in a well, showed CPE within 120 h, it was regarded as positive, which means the single-cell contained FMDV particles.

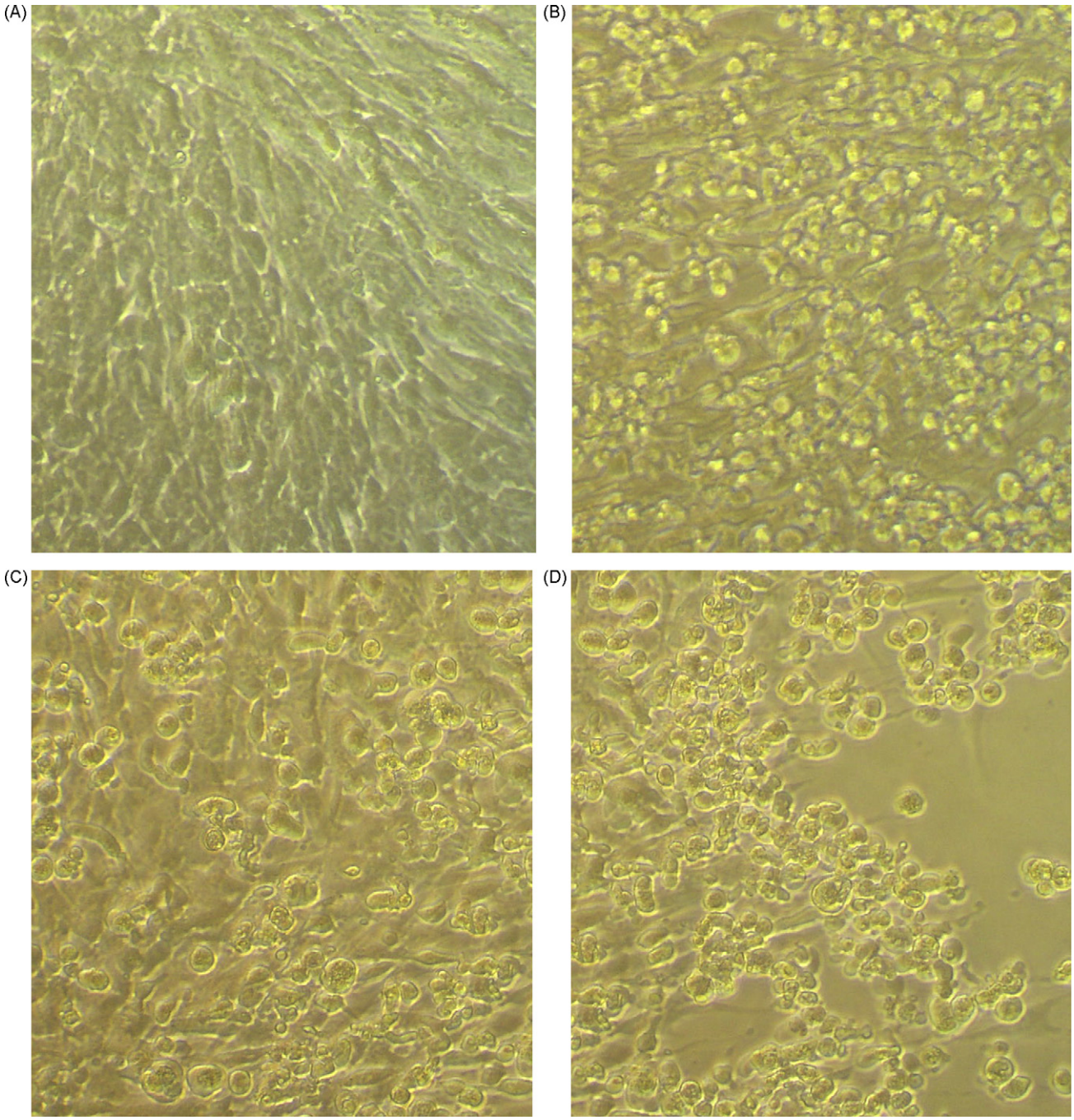

Fig. 5. Morphological features of BHK-21 cells infected with FMDV (200×). (A) Negative control cells, (B) 18 h post-infection, (C) 24 h post-infection and (D) 30 h post-infection.

effects (30 h.p.i.). According to these results, the maximum ratio of virulence-positive cells (76.3%) appeared at 24 h.p.i. It is likely that there is an upper limit for the positive ratio in virulence-positive cells.

3.5. Single-cell real-time RT-PCR

Based on the results in Section 3.4, the conditions for single-cell isolation for the qRT-PCR assay was as follows: 100 PFU FMDV was used to infect cells and 24 h later single cells were isolated and examined by qRT-PCR.

In total, 224 single-cells were isolated and run on sc-qRT-PCR. Of these, 185 single-cell samples contained more than 10 copies of viral RNA (ranging from several to thousands, up to 1 000 000), and were considered to be positive. In contrast, 39 single-cell samples were considered to be negative (Fig. 6). The ratio of viral RNA positive to negative cells was 82.6%. Most positive samples carried viral RNA copies ranging from 1000 to 10 000.

4. Discussion

Single-cell PCR, including sc-qRT-PCR, came into use in the late 1980s, and have subsequently been validated to work well with both DNA and mRNA in single-cell samples. It was generally accepted that the difficulties in performing single-cell PCR were partly due to difficulties in isolating and lysing single-cell samples,

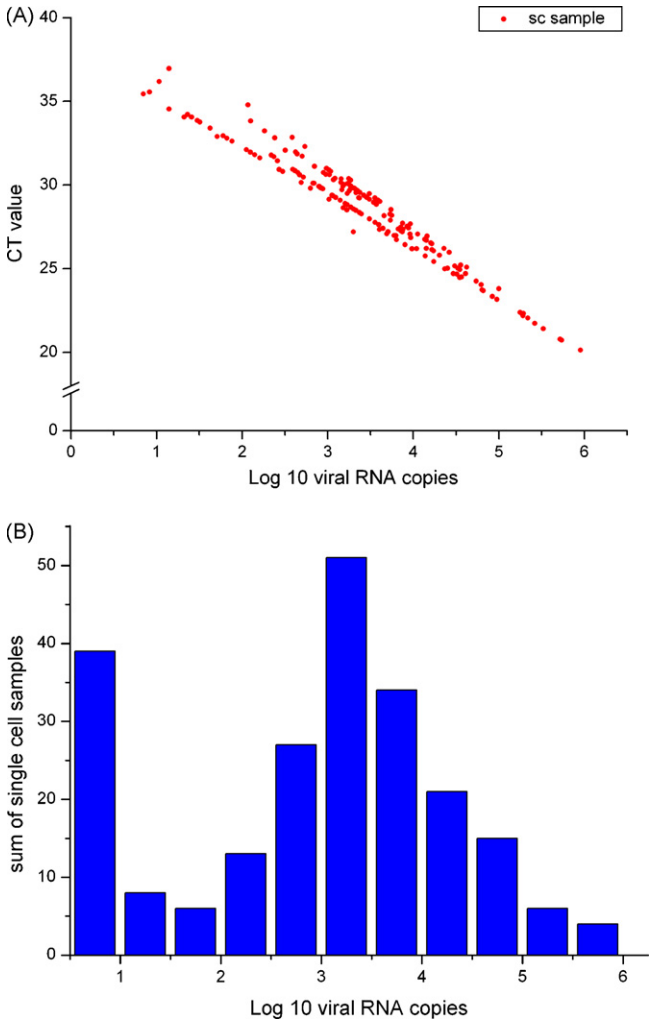

Fig. 6. Results of sc-qRT-PCR assays. (A) The scattered distribution of 185 single-cell samples that carried different numbers of FMDV RNA copies (one red dot represents a single-cell sample). The dots retained good linearity. (B) Grouping of 224 single-cell samples according to viral RNA copies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

especially for single-cell RT-PCR assays. Liu et al. (2004) introduced single-cell cytoplasm into a pipette containing 3 μl 0.9% NaCl solution and 6 U RNase inhibitor. Meanwhile, Parhar et al. (2003) digested single cells with 1 μg proteinase K and 10 U ribonuclease inhibitor at 53 °C for 1 h after cell lysis. Other researchers have used lysis buffer or QuantiLyse buffer for cell lysis (Alsbo et al., 2001; Pierce et al., 2002; Rice et al., 2002; Wagatsuma et al., 2005). In the present study, different methods were used to optimize the process of cell lysis. In conclusion, the method described above was considered to be reasonable and feasible. Samples were heat-denatured at 98 °C for 3 min, digested with 1 μg of proteinase K and 10 U ribonuclease inhibitor at 53 °C for 1 h and then frozen. Freeze–thaw and protease-based lysis were used in the assay for cell lysis after heat denaturation. Little differences were found between these two methods of cell lysis, which is consistent with that of Pierce et al. (2002 and 2003) (data not shown). To minimize the influence of proteins on PCR, protease-based lysis was preferred and chosen for pretreatment of single-cell samples. Standard RNA templates were introduced as control RNAs to confirm that the RNA did not degrade during cell lysis. Furthermore, RNase*Zap* Solution (Ambion, U.S.A.) was used to clean the work surfaces and lab apparatus at the beginning of every experiment to remove RNase contamination.

FMDV is a positive-sense, single-stranded RNA virus, which can cause severe acute infection of BHK-21 cell lines. The virus infectivity assay was introduced to identify the peak-time of virus replication and release in its life cycle. Single acutely infected cells were isolated and added to the supernatant of normal BHK-21 cells growing in a 96-well plate (one cell per well). Approximately 80 h later, mature virions were released from the single-cell and then caused acute infection that can be observed easily under a microscope. The results of the virus infectivity assay indicated that the maximum ratio of virulence-positive cells was at 24 h.p.i. In these conditions, it was possible to identify the maximum number of viral RNA copies that one cell can endure. Surprisingly, in one cell the largest number of viral genome RNA copies reached 900 000, which may be the upper limit of viral load that one cell can endure before lysis of the cell membrane and release of virions. The maximum ratio of virulence-positive cells was 76.3%, which is slightly less than that of viral RNA positive cells (82.6%). These results indicate that cells containing the FMDV genomic RNA were not always lysed and did not release viral particles. It is likely that there is a threshold lower limit of viral RNA copies in one cell to cause lysis. Considering this, the results of the virus infectivity assay and sc-qRT-PCR in this study were consistent.

Since this was the first time that sc-qRT-PCR had been performed for viral RNA quantitation rather than host mRNA, which caused some uncertainty whether every single host cell would be infected, the GAPDH gene was used as a control gene for the conventional single-cell RT-PCR to confirm the feasibility of single-cell isolation and pretreatment. Although the amounts of GAPDH differed between single-cell samples, the threshold for detection was 0.2% of the content of one cell, which was found to be reliable and reproducible. Thus, including GAPDH as a control can limit the emergence of false negative results.

In the present study, sc-qRT-PCR of the viral genome RNA was established, and was sensitive for detection of viral RNA copies greater than 10 per cell. Previous studies on the time-course of viral replication were based on a large number of cells or a block of tissues and samples were obtained at different times post-infection (Gu et al., 2007). However, sc-qRT-PCR offers a new method for this kind of research, because the viral loads in a single-cell differs substantially between cells in a certain population (e.g. T-25 flask) at any time after inoculation of FMDV. In the assay, viral RNA copies in one cell were different from the others in 185 positive samples, and ranged from several to millions. One reason for this finding is that the efficiency of viral RNA replication differs between cells. In addition, the starting time of viral RNA replication varies between cells, which results in differing accumulation of viral RNA. The grouping and distribution of FMDV RNA copies in the 224 single-cell samples broadly reflects the process of genome RNA replication. Furthermore, sc-qRT-PCR can be combined with plaque assays to better clarify the latent period of the viral one-step growth curve to calculate the negative ratio.

Little is known about the mechanism of FMDV replication. Nevertheless, it has been suggested that a negative-sense RNA intermediate is produced and used as a template for the synthesis of new single-stranded positive-sense RNA molecules during FMDV replication (Cleaves et al., 1981). (1) Research on quantitation of negative-sense RNA was carried on in our laboratory using sc-qRT-PCR assay, focusing on the ratio of positive-sense to negative-sense RNA copies, which represents the replication level (data not shown). (2) Although FMDV is highly cytolytic, it can also establish persistent infections not only in live animals but also in cell cultures. This new sc-qRT-PCR assay was used to quantify FMDV RNA copies in single persistently infected cells (data not shown).

To our knowledge, this is the first report to use sc-qRT-PCR for detection and quantitation of viral genomic RNA rather than host

mRNA. The methods for isolation and pretreatment of single cells are universal and can be used in other cell lines whether inoculated with viruses or not.

Acknowledgements

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References

Alexandersen, S., Mowat, G.N., 2005. Foot-and-mouth disease: host range and pathogenesis. Curr. Top. Microbiol. Immunol. 288, 9–42.

Alexandersen, S., Zhang, Z., Donaldson, A.I., Garland, A.J., 2003. The pathogenesis and diagnosis of foot-and-mouth disease. J. Comp. Pathol. 129, 1–36.

Alsbo, C.W., Wrang, M.L., Moller, F., Diemer, N.H., 2001. Is the AMPA receptor subunit GluR2 mRNA an early indicator of cell fate after ischemia? A quantitative single cell RT-PCR study. Brain Res. 894, 101–108.

Belsham, G.J., 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family: aspects of virus protein synthesis, protein processing and structure. Prog. Biophys. Mol. Bio. 60, 241–260.

Callahan, J.D., Brown, F., Osorio, F.A., Sur, J.H., Kramer, E., Long, G.W., Lubroth, J., Ellis, S.J., Shoulars, K.S., Gaffney, K.L., Rock, D.L., Nelson, W.M., 2002. Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. J. Am. Vet. Med. Assoc. 220, 1636–1642.

Cleaves, G.R., Ryan, T.E., Schlesinger, R.W., 1981. Identification and characterization of type 2 dengue virus replicative intermediate and replicative form RNAs. Virology 111, 73–83.

De la Torre, J.C., Dávila, M., Sobrino, F., Ortín, J., Domingo, E., 1985. Establishment of cell lines persistently infected with foot-and-mouth disease virus. Virology 145, 24–35.

Domingo, E., Escarmís, C., Baranowski, E., Ruiz-Jarabo, C.M., Carrillo, E., Núñez, J.I., Sobrino, F., 2003. Evolution of foot-and-mouth disease virus. Virus Res. 91, 47–63.

Fosgate, G.T., Tavornpanich, S., Hunter, D., Pugh, R., Sterle, J.A., Schumann, K.R., Eberling, A.J., Beckham, T.R., Martin, B.M., Clarke, N.P., Adams, L.G., 2008. Diagnostic specificity of a real-time RT-PCR in cattle for foot-and-mouth disease and swine for foot-and-mouth disease and classical swine fever based on non-invasive specimen collection. Vet. Microbiol. (Epub ahead of print).

Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. Clin. Microbiol. Rev. 17, 465–493.

Gu, C., Zheng, C., Wu, J., Qu, S., Yang, W., Chang, H., 2003. Rapid selection of the cells persistently infected with foot-and-mouth disease virus and the study of natures in persistent infection cells. Virologica Siniga 18, 459–463.

Gu, C., Zheng, C., Shi, L., Zhang, Q., Li, Y., Lu, B., Xiong, Y., Qu, S., Shao, J., Chang, H., 2007. Plus- and minus-stranded foot-and-mouth disease virus RNA quantified simultaneously using a novel real-time RT-PCR. Virus Genes 34, 2461–2469.

Hartshorn, C., Anshelevich, A., Wangh, L.J., 2005. Rapid, single-tube method for quantitative preparation and analysis of RNA and DNA in samples as small as one cell. BMC Biotechnol. 5, 2.

Hartshorn, C., Eckert, J.J., Hartung, O., Wangh, L.J., 2007. Single-cell duplex RT-LATE-PCR reveals Oct4 and Xist RNA gradients in 8-cell embryos. BMC Biotechnol. 7, 87.

Hillman, K.L., Knudson, C.A., Carr, P.A., Doze, V.A., Porter, J.E., 2005. Adrenergic receptor characterization of CA1 hippocampal neurons using real time single cell RT-PCR. Mol. Brain. Res. 139, 267–276.

Hofmann, M.A., Thür, B., Liu, L., Gerber, M., Stettler, P., Moser, C., Bossy, S., 2000. Rescue of infectious classical swine fever and foot-and-mouth disease virus by RNA transfection and virus detection by RT-PCR after extended storage of samples in Trizol. J. Virol. Methods 87, 29–39.

Horsington, J., Zhang, Z., 2007. Analysis of foot-and-mouth disease virus replication using strand-specific quantitative RT-PCR. J. Virol. Methods 144, 149–155.

Jemiolo, B., Trappe, S., 2004. Single muscle fiber gene expression in human skeletal muscle: validation of internal control with exercise. Biochem. Biophys. Res. Commun. 320, 1043–1050.

Jeffreys, A.J., Wilson, V., Neumann, R., Keytel, J., 1988. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic. Acids. Res. 16, 10953–10971.

Krapf, R., Solioz, M., 1991. Na/H antiporter mRNA expression in single nephron segments of rat kidney cortex. J. Clin. Invest. 88, 783–788.

King, D.P., Ferris, N.P., Shaw, A.E., Reid, S.M., Hutchings, G.H., Giuffre, A.C., Robida, J.M., Callahan, J.D., Nelson, W.M., Beckham, T.R., 2006. Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time reverse transcription polymerase chain reaction assays. J. Vet. Diagn. Invest. 18, 93–97.

Kullberg, M., Nilsson, M.A., Arnason, U., Harley, E.H., Janke, A., 2006. Housekeeping genes for phylogenetic analysis of Eutherian relationships. Mol. Biol. Evol. 23, 1493–1503.

Liu, B., Naoki, H., Jiang, B., Yasuhisa, N., Zhang, N., Wu, B., Kaori, K., Megumi, T., Hiroko, M., Chiyoko, I., 2004. Single cell RT-PCR demonstrates differential expression of $GABA_C$ receptor ρ subunits in rat hippocampal pyramidal and granule cells. Mol. Brain Res. 123, 1–6.

Mattano Jr., L.A., Moss, T.J., Emerson, S.J., 1992. Sensitive detection of rare circulating neuroblastoma cells by the reverse transcriptase polymerase chain reaction. Cancer. Res. 52, 4701–4705.

Moss, A., Hass, B., 1999. Comparison of the plaque test and reverse transcription nested PCR for detection of FMDV in nasal swabs and probing samples. J. Virol. Methods 80, 59–67.

Parhar, I.S., Ogawa, S., Hamada, T., Sakuma, Y., 2003. Single-cell real-time quantitative polymerase chain reaction of immunofluorescently identified neurons of gonadotropin-releasing hormone subtypes in cichlid fish. Endocrinology 144, 3297–3300.

Pierce, K.E., Rice, J.E., Sanchez, J.A., Wangh, L.J., 2002. QuantiLyse™: reliable DNA amplification from single cells. BioTechniques 32, 1106–1111.

Pierce, K.E., Rice, J.E., Sanchez, J.A., Wangh, L.J., 2003. Detection of cystic fibrosis alleles from single cells using molecular beacons and a novel method of asymmetric real-time PCR. Mol. Hum. Reprod. 9, 815–820.

Ponnaiya, B., Baker, G.J., Pherson, G.R., Geard, C.R., 2007. Quantifying a bystander response following microbeam irradiation using single-cell RT-PCR analyses. Exp. Hematol. 35, 64–68.

Rice, J.E., Sanchez, J.A., Pierce, K.E., Wangh, L.J., 2002. Real-time PCR with molecular beacons provides a highly accurate assay for detection of Tay-Sachs alleles in single cells. Prenat. Diagn. 22, 1130–1134.

Shaw, A.E., Reid, S.M., Ebert, K., Hutchings, G.H., Ferris, N.P., King, D.P., 2007. Implementation of a one-step real-time RT-PCR protocol for diagnosis of foot-and-mouth disease. J. Virol. Methods 143, 81–85.

Verheyden, B., Lauwers, S., Rombaut, B., 2003. Quantitative RT-PCR ELISA to determine the amount and ratio of positive- and negative-strand viral RNA synthesis and the effect of guanidine in poliovirus infected cells. J. Pharm. Biomed. Anal. 33, 303–308.

Wacker, M.J., Tehel, M.M., Gallagher, P.M., 2008. Technique for quantitative RT-PCR analysis directly from single muscle fibers. J. Appl. Physiol. (Epub ahead of print).

Wagatsuma, A., Sadamoto, H., Kitahashi, T., Lukowiak, K., Urano, A., Ito, E., 2005. Determination of the exact copy numbers of particular mRNAs in a single cell by quantitative real-time RT-PCR. J. Exp. Biol. 208, 2389–2398.