Research article

Assessment of the sensitivity of primary cells and cell lines to the Southern African Territories (SAT) serotypes in the diagnosis of foot-and-mouth disease virus

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A B S T R A C T

Virus isolation is the gold standard for foot-and-mouth disease virus (FMDV) detection in diagnostic procedures. This technique is heavily reliant on the use of sensitive cells for rapid and accurate detection of FMDV. To investigate the sensitivity of RM (primary lamb kidney cells), BHK-21 (baby hamster kidney cells) and IR-P1 (a derivative of female pig kidney cells) to infection with FMDV of the Southern African Territories (SAT) serotypes, we examined the virus concentration required to induce cytopathic effect (CPE) on each cell type. The results suggested that sensitivity of RM and IR-P1 cells was high and not significantly different ($P < 0.05$). BHK-21 however, exhibited low sensitivity to the strains used. Comparisons of three batches of each cell type were also done to establish the consistency of the sensitivity of these cells to FMDV infection. IR-P1 and BHK-21 cell batches gave consistent results for all samples used whereas RM cells showed significant differences ($P > 0.05$) between batches. TCID 50/ml was used to determine the viral titre required to induce CPE. IR-P1 cell line proved to have consistently higher TCID50/mL for all cell batches while RM cell batches displayed a difference in TCID50/mL values. The IR-P1 cell line was concluded to be a good cell culture system for virus isolation as it showed relatively high and reproducible sensitivity to all the FMDV strains used. The findings of this study indicate that the use of IR-P1 cell line could be considered for FMDV diagnostic work.

1. Introduction

Foot-and-mouth disease (FMD) is one of the most contagious livestock diseases which bring about economic losses to many countries that depend on livestock for generation of revenue. Taking Botswana as an example, livestock production, particularly beef products, contribute up to 65% of revenue in the agricultural sector (Seleka and Kebakile, 2015). Botswana exports approximately 80% of its beef to the European and South African markets (van Engelen et al., 2013). The revenue gained from these transactions account for up to one third of the GDP of the country. An uncontrolled FMDV outbreak can therefore cause a decline in income because as a control measure, international markets do not accept any beef imports during an FMDV outbreak. Early detection of FMDV and initiation of control measures are thus pivotal in circumvention of severe economic losses.

Clinical signs are the first diagnosis of FMDV infection. The primary clinical signs of this disease are fever and lesions on the tongue, feet, snout, and teats (Grubman and Baxt, 2004). The resulting secondary clinical signs may include lameness, depression, extreme salivation, loss of appetite and subsequent weight loss, growth retardation and a decrease in milk production. Upon witnessing clinical signs, laboratory diagnostic tests must be performed to confirm the presence of the virus. The diagnostic techniques recommended by OIE include virus isolation, enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription PCR (real time RT-PCR) assays. Although real-time RT-PCR is the most sensitive and rapid test, virus isolation is still regarded as the gold standard for FMDV diagnosis because it detects 'live' virus, and generates higher virus titres for downstream testing, such as serotyping ELISA (Jamal and Belsham, 2013).

Virus isolation procedures employ the use of cells for propagation of the virus, thus there is a need to utilize cells which are highly sensitive to

the relevant FMDV serotypes. Primary bovine thyroid (BTY) cells have been identified as the most sensitive cells to FMDV infection (House and Yedloutschnig, 1982). Albeit being immensely sensitive, primary BTY cells tend to lose sensitivity when passaged and frozen (Fukai et al., 2015). This makes these cells inapt for rapid diagnostics because fresh cells from thyroid tissue have to be available each time for virus isolation, which is expensive, time consuming and laborious. A study by Brehm et al. (2009) showed that a goat fetal tongue cell line (ZZR-127) has sensitivity equivalent to BTY cells and there are other studies that have been performed using this cell line that also support Brehm et al.'s findings (Fukai et al., 2013, 2015). Another cell line with reputable FMDV sensitivity is the fetal porcine kidney cell line (LFBK-$\alpha_V\beta_6$). The sensitivity of this cell line is similar to the ZZR-127 cell line (LaRocco et al., 2013; Fukai et al., 2015), however, our laboratory has not been able to easily acquire both cell lines.

The OIE reference laboratory in Botswana currently employs primary lamb kidney (RM) cells for the virus isolation procedure for diagnosing FMDV. However, these cells have shown a tendency to produce results which are variable in sensitivity to FMDV from one cell batch to the other. This study therefore aimed to assess the possibility of a transition to the use of continuous cell lines in diagnosis of FMDV. This was done by determining the sensitivity of cell lines to FMDV Southern African Territories (SAT) serotypes and determining reproducibility of infection of cells by FMDV. Primary lamb kidney cells (RM) were used in this study because these cells are used for virus isolation in the OIE reference in Botswana. A derivative of female pig kidney cells (IR-P1) was also investigated because these cells are related to IBRS-2 cells which have been reported in literature to be sensitive to FMDV infection (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2018; Jamal and Belsham, 2013; Paprocka, 2008a,b). Limited data areavailable on the IR-P1 cell line, therefore this study is the first published data on the use of this cell line in FMDV diagnostics. The baby hamster kidney cells (BHK-21) were also used because these cells are mainly used in propagation of the FMDV virus during vaccine production, and therefore demonstrate a degree of sensitivity to FMDV infection (Zabal and Fondevila, 2013).

2. Materials and methods

2.1. Cell preparations

Three batches of RM, IR-P1 (Merial, France) and BHK-21 were cultured in 48 well plates (Nunc MicroWell Thermo Fisher). RM cells were cultured in RM media; Minimum Essential Medium ((MEM), Thermo Fisher) 5% new born calf serum (Gibco), 20U/mL penicillin (Gibco), 2.5% lactalbumin hydrolysate (Gibco) and sodium bicarbonate (Sigma Aldrich) at pH 7.4 to 7.6) and a seeding density of $1.5x10^5$ cells/mL. IR-P1 cells were propagated in IR-P1 media; MEM (Thermo Fischer), 10% new born calf serum (Gibco), 20U/mL penicillin (Gibco), 2.5% lactalbumin hydrolysate; (Gibco) and sodium bicarbonate (Sigma Aldrich) at pH 7.4 to 7.6) and a seeding density of $2x10^5$ cells/mL. BHK-21 cells were propagated in complete growth medium for BHK cells; alpha MEM (Thermo Fisher) 2 mM-glutamine (Gibco), 20U/mL penicillin (Gibco) and 5% fetal bovine serum (Gibco), at pH 7.2 to 7.4 and a seeding density of $7.5x10^5$ cells/mL.

Each well of the 48 well plates was inoculated with 200 μL of the cell suspension. The plates were then incubated at 37 °C in a 5% CO_2 incubator (Thermo Scientific. Jouan). The plates were monitored daily under the microscope for confluence (Olympus, Model: CKX 31). Cells were infected at 90–100% confluence.

2.2. Virus samples

FMDV samples representing SAT 1, 2 and 3 were obtained from the collection in the OIE FMD Regional Reference Laboratory for Sub-Saharan Africa in Gaborone, Botswana (OIE FMD RRLSSA). The following samples were used: SAT1/BOT11/2015, SAT2/BOT2/2018 and SAT3/ZAM7/2018.

2.3. TCID50/mL assay

The endpoint dilution assay 50% tissue culture infective dose (TCID50/mL) was employed to determine the sensitivity of cells to viral infection. This assay defines the amount of virus concentration required to kill 50% of the cell population. To establish the consistency of FMDV infection, three batches of each cell type were infected with different dilutions of field viruses (SAT1/BOT11/2015, SAT2/BOT2/2018 and SAT3/ZAM7/2018) in a 48 well plate and then monitored for CPE after incubation. To achieve this, ten-fold dilutions of each of the virus strains were done in the different media for the three cell types. This procedure was performed in duplicate each virus strain The dilutions of 10^{-2} - 10^{-7} were used to infect three batches of confluent cells of each cell type grown in 48 well plates. Each column of the plate was assigned a specific dilution. For each dilution, 6 wells in a row were infected and two were not infected, these 2 wells served as negative controls. The plates were incubated at 37 °C in 5% CO_2 (Thermo Scientific. Jouan). The cells were examined for cytopathic effect (CPE) under the light microscope (Olympus, Model: CKX 31) after 24 h and 48 h of infection. This procedure was performed in duplicate for each cell type. Wells showing CPE were recorded and TCID 50/mL was then calculated using the Reed-Muench method (1983). The titers of each cell type were expressed as log of the TCID50/mL.

2.4. Statistical analysis

Data obtained from the TCID50/mL assay were analyzed using one way analysis of variance (ANOVA). The resultant data was then compared using the multiple comparison Tukey's range test in GraphPad Prism (version 8.3. 0, California, USA). This statistical test was done to establish if there is a significant difference ($P < 0.05$) in sensitivity within cell batches of the same cell type. The same test was also used to assess any difference in sensitivity between the selected cell types.

3. Results

FMDV infected cells exhibit morphological changes termed cytopathic effect (CPE), commonly characterized by disorganization of internal cellular membranes, cell rounding and detachment from cell monolayer due to cell death (Kamal et al., 2014). Monolayers of cells exhibiting CPE in RM cells, IR-P1cells and BHK-21 cells are shown in Figure 1.

Different dilutions of three SAT virus serotypes were used for infection of the cells types under investigation. In instances where virus dilution was low (10^{-2} and 10^{-3}) for all tested samples, all three IR-P1 cell batches, had the most wells showing CPE regardless of virus serotype (Figure 2). RM cells had comparable results with IR-P1 but batch 1 consistently had a lower number of wells showing CPE. All three batches of BHK-21 had lower number of wells showing CPE at low virus dilutions when compared to IR-P1 cells. Over the full range of dilutions used, the IR-P1 batches were able to detect low virus concentrations. RM batch 2 and 3 cells were also able to detect low virus concentrations, but this was not reproducible in RM batch 1 cells. Thus RM cells showed inconsistency in sensitivity to the FMDV infection (Figure 2). Compared to IR-P1 and RM cells, all the BHK-21 cells batches consistently showed lower sensitivity to infection with FMDV. The BHK-21 cells were less sensitive to infection as these cells required low virus dilutions (Figure 2) to exhibit CPE, as observed in all the cell batches. Both cell lines used in this study showed a consistent sensitivity to FMDV infection by all the strains used.

The Reed Muench method was used to calculate the titres required to induce CPE in 50% of the infected wells. This was done by taking the cumulative number of wells showing CPE per dilution over the total number of wells infected. This value was used to calculate the
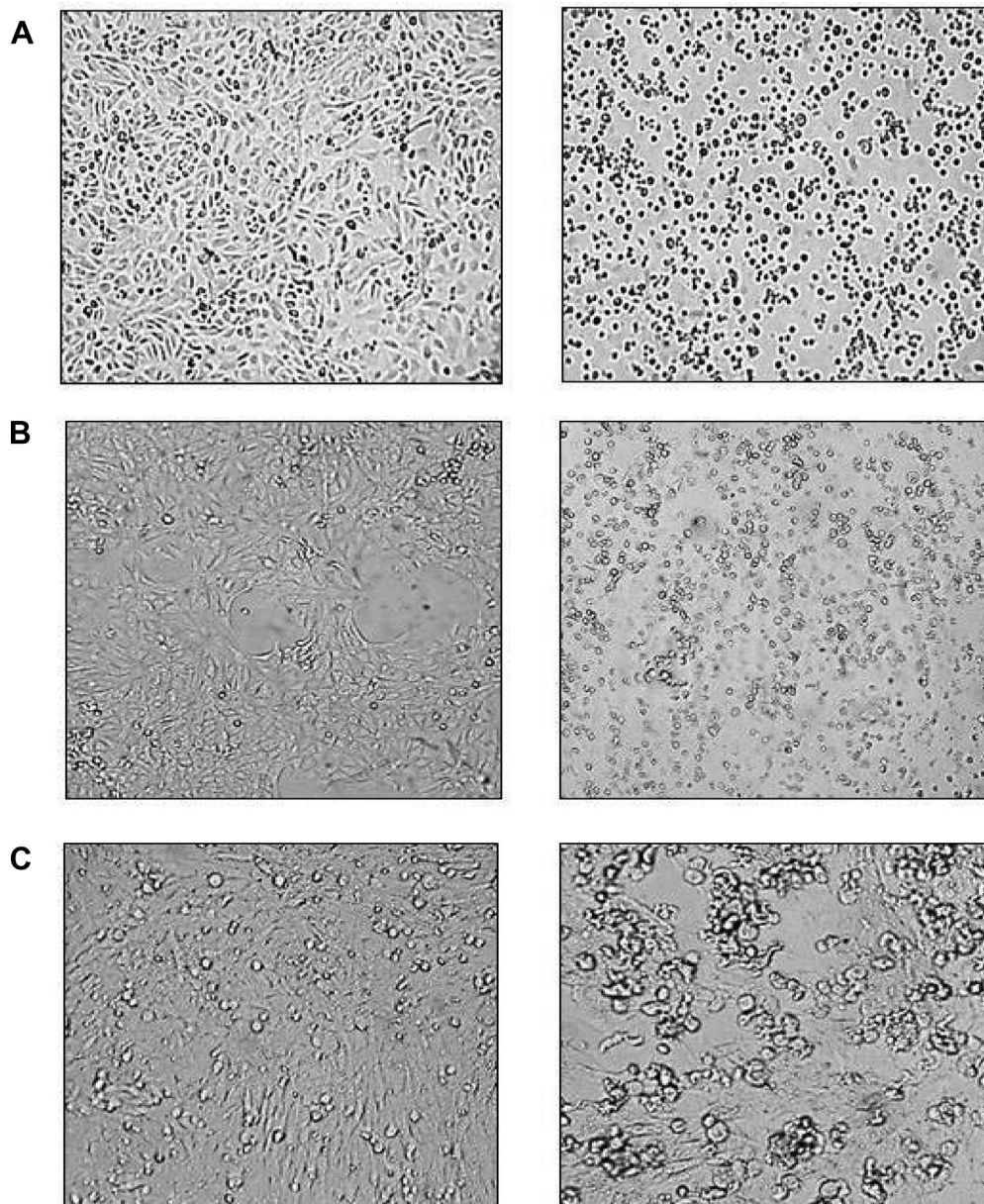
Figure 1. Uninfected cell monolayers of the three cell types, each with approximately 90%–100% cell confluency (**left**) and cells with 100% cell CPE (**right**) as observed under light microscope. A) RM cells in culture before infection (left) and after infection (right) with FMDV. B) IR-P1 cell line in culture before and after infection (left and right respectively C) BHK-21 cell line monolayers before infection with FMDV (left) and after infection with FMDV (right).

logarithmic TCID50/mL. The reciprocal of the logTCID50/mL represents the infectious dose per unit volume (Reed and Muench, 1983). The TCID50/mL for IR-P1 and RM cells was relatively high (Figure 3). However, the P value from the ANOVA analysis, showed that the average TCID50/mL were similar between IR-P1 and RM ($P > 0.05$). BHK-21 exhibited a low TCID50/mL, which was expected given the low virus dilution required to induce CPE (Figure 2). The ANOVA analysis of the TCID50/mL value of BHK-21 against RM and IR-P1 showed that the difference in these TCID50/mL values is statistically significant ($P < 0.05$).

4. Discussion

In previous studies (LaRocco et al., 2013; Paprocka, 2008a,b), RM cells have been reported to have a relatively high sensitivity to FMDV infection. This sensitivity has also been observed in practical terms in various OIE laboratories, including the one in Botswana. Although

sensitive, these cells have shown an inconsistency in sensitivity to FMDV from one cell batch to another, a problem experienced by most primary cells (Fukai et al., 2015). The variation observed between the primary cell line batches may arise because different batches of primary cells are derived from different specimen, hence these cells tend to differ in sensitivity depending on the genetics and age of the animal from which the tissue was derived (Kaur and Dufour, 2012). Cell lines therefore are more likely to provide consistency because these cells are clones of one parent cell which has undergone continuous passages over time and therefore have developed homogenous genotypic and phenotypic traits (Kaur and Dufour, 2012). It must be noted however that the performance of cell lines for diagnostic purposes needs to be monitored as extended passaging can change the properties and hence sensitivity of the cells.

IR-P1 cell line has shown a consistently higher level of sensitivity to FMDV infection as compared to another cell line (BHK-21) and comparable sensitivity with the primary cells (RM). The findings of this study
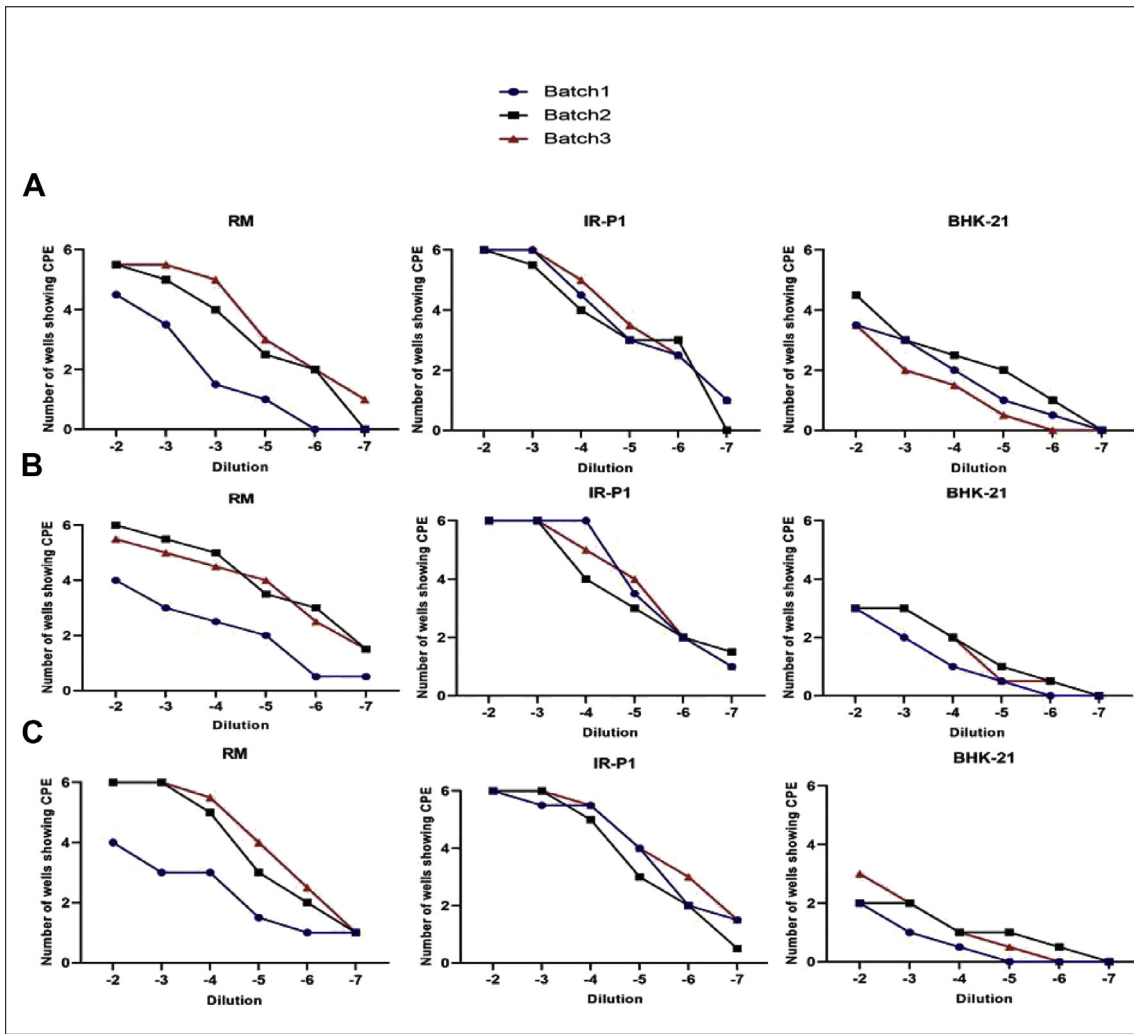
Figure 2. Comparison of three cell batches of RM, IR-P1 and BHK-21 infected with: A) SAT1/BOT11/2015, B) SAT2/BOT2/2018, and C) SAT3/ZAM7/2018 over different viral dilutions.
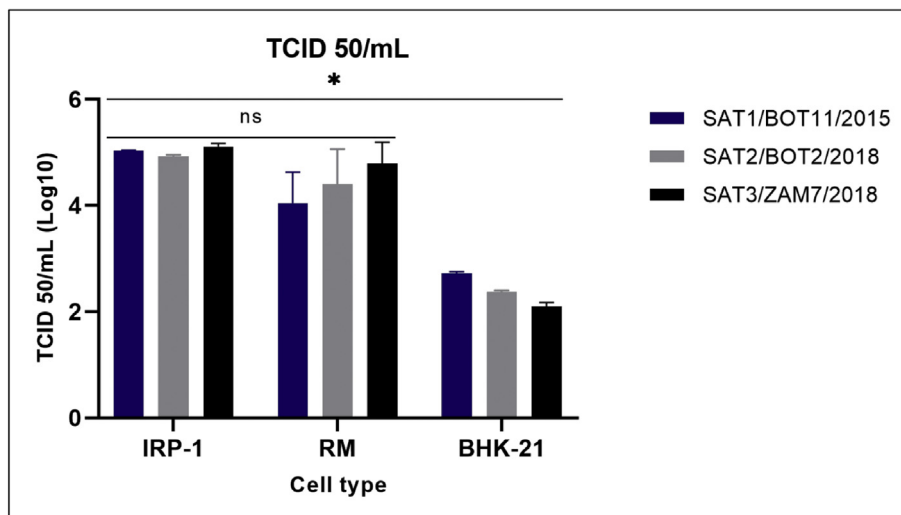

Figure 3. Graphical comparison of the TCID50/mL assay used the three cell types infected with SAT1/BOT11/2015, SAT2/BOT2/2018 and SAT3/ZAM7/2018.

were contrary to what was expected. According to the Manual for Diagnostic Tests and Vaccines for Terrestrial Animals (2018) primary cells such as RM cells are known to have high sensitivity than cell lines. IBRS-2 which is a derivative to the IR-P1 cell line used in this study has been reported to have a lower sensitivity than most primary cells (LaRocco et al., 2013; Longjam et al., 2011). It was therefore unexpected that the IR-P1 cells had comparable sensitivity to primary lamb kidney cells. It is important to highlight that most work has been done on IBRS-2 cells but not IR-P1 cells. Therefore, the findings of this study provide the first report of the probable utility of this cell line in FMDV diagnosis. Further studies on this cell line can therefore be undertaken in the future to determine the life cycle of the virus in these cells and the factors that determine the sensitivity of these cells to FMDV infection such as cell surface receptors and cell culture conditions. This additional work would be invaluable for validation of a probable transition to the use of cell lines in routine diagnosis of FMD. Characterization of performance of the cell line in terms of long-term sensitivity and specificity, as well as quantitative measures of detection limits is required prior to its adoption for routine diagnostic use.

5. Conclusion

The overall aim of this study was to compare the sensitivity of RM, IRP1 and BHK-21 cells to FMDV infection using SAT serotypes. Our study showed that the IR-P1 cell line can provide a more reliable and cost-effective system for virus isolation procedures in absence of LFBK-$\alpha_V\beta_6$ and ZZ-R127. The overall the sensitivity of RM and IR-P1 cells is similar, but there is less batch-to-batch variation with the IR-P1 cells.

IR-P1 cells are also easier to source from culture banks as opposed to relying on fresh specimen for primary culture. The combination of high sensitivity, consistency to infection and easy access therefore make the IRP1 cell line an ideal cell culture system over RM cells and BHK-21.

Declarations

Author contribution statement

T. Kabelo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

E. Fana: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

K. Lebani: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

References

Brehm, K.E., Ferris, N.P., Lenk, M., Riebe, R., Haas, B., 2009. Highly sensitive fetal goat tongue cell line for detection and isolation of foot-and-mouth disease virus. J. Clin. Microbiol. 47 (10), 3156–3160. https://doi.org/10.1128/jcm.00510-09.

Fukai, K., Morioka, K., Yamada, M., Nishi, T., Yoshida, K., Kitano, R., Yamazoe, R., Kanno, T., 2015. Comparative performance of fetal goat tongue cell line ZZ-R 127 and fetal porcine kidney cell line LFBK-$\alpha\nu\beta6$ for Foot-and-mouth disease virus isolation. J. Vet. Diagn. Invest. 27, 516–521.

Fukai, K., Onozato, H., Kitano, R., Yamazoe, R., Morioka, K., Yamada, M., Ohashi, S., Yoshida, K., Kanno, T., 2013. Availability of a fetal goat tongue cell line ZZ-R 127 for isolation of Foot-and-mouth disease virus (FMDV) from clinical samples collected from animals experimentally infected with FMDV. J. Vet. Diagn. Invest. 25, 770–774.

Grubman, M.J., Baxt, B., 2004. Foot-and-Mouth disease. Foot Mouth Dis. 17 (2), 465–493.

House, J.A., Yedloutschnig, R.J., 1982. Sensitivity of seven different types of cell cultures to three serotypes of foot-and-mouth disease virus. Comp. Med. 46 (2), 186–189.

Jamal, S.M., Belsham, G.J., 2013. Foot-and-mouth disease: past, present and future. Vet. Res. 44, 116. https://doi.org/10.1186/1297-9716-44-116 (accessed23/02/18.

Kamal, T., Naeem, K., Munir, A., Ali, M., Ullah, A., 2014. Comparative study for the sensitivity of BHK-21 and bovine kidney cell line for the isolation of FMD viruses. In: Proceedings of 2014 11th International Bhurban Conference on Applied Sciences & Technology (IBCAST) Islamabad, Pakistan, 14th - 18th January, 2014.

Kaur, G., Dufour, J.M., 2012. Cell lines. Spermatogenesis 2 (1), 1–5.

LaRocco, M., Krug, P.W., Kramer, E., Ahmed, Z., Pacheco, J.M., Duque, H., Baxt, B., Rodriguez, L.L., 2013. A continuous bovine kidney cell line constitutively expressing bovine $\alpha_V\beta_6$ integrin has increased susceptibility to foot-and-mouth disease virus. J. Clin. Microbiol. 53, 775.

Longjam, N., Deb, R., Sarmah, A.K., Tayo, T., Awachat, V.B., Saxena, V.K., 2011. A brief review on diagnosis of foot-and-mouth disease of livestock: conventional to molecular tools. Vet. Med. Int. 2011, 1–17.

Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2018 [WWW Document], n.d. [WWW Document]. OIE. URL. http://www.oie.int/standard-setting/terrestrial-manual/access-online/ (accessed 23.02.2019).

Paprocka, G., 2008a. Isolation and identification of thefoot and mouth disease virus. Med. Weter. 6 (12), 1404–1406.

Paprocka, G., 2008b. Applying primary lamb kidney cell culturein diagnosing foot and mouth disease. Med. Weter. 64 (3), 332–334.

Reed, L., Muench, H., 1938. A simple method of estimating fifty per cent Endpoints12. Am. J. Epidemiol. 27, 493–497.

Seleka, T.B, Kebakile, P.G, 2015. Export Competitiveness of Botswana's Beef Industry. SSRN Electron. J. 31 (1), 76–101.

van Engelen, A., Malope, P., Keyser, J., Neven, D., 2013. Botswana Agrifood Value Chain Project. Beef Value Chain Study. Food and Agriculture Organization of the United Nations & Ministry of Agriculture, Botswana.

Zabal, O., Fondevila, N., 2013. Selection of highly susceptible cell lines to foot and mouth disease virus infection. Open J. Vet. Med. 3, 263–266.