



STUDIES ON CULTURAL CHARACTERISTICS OF CONTAGIOUS ECTHYMA (ORF) VIRUS

L. IVANOV, M. HRISTOV & R. PESHEV

National Diagnostic and Research Veterinary Medical Institute, Sofia, Bulgaria

Summary

Ivanov, L., M. Hristov & R. Peshev, 2016. Studies on cultural characteristics of contagious ecthyma (Orf) virus. *Bulg. J. Vet. Med.*, 19, No 4, 308–316.

Ecthyma contagiosum is a viral disease of sheep and goats, which is caused by *Parapoxvirus*. Primary cell cultures of lamb testis, lamb kidney, rabbit kidney and permanent cell lines: foetal sheep kidney (FSK), sheep foetal thymus (SFT), sheep foetal muscle (SFM), sheep plexus choroideus (SPC), bovine trachea (BTR), embryonic bovine trachea (EBTR), Madin Darby bovine kidney (MDBK) and baby hamster kidney (BHK 21) were used. For inoculation of the cell cultures, 10% suspensions of scabs in PBS or nasal swabs from Orf infected sheep and goats were used. The vaccinal strain Phylaxia was used as reference. Reproduction of isolates was controlled daily by assessment of the cytopathic effect and dynamics was monitored on 5 types of cell cultures after 10 consecutive passages. Intracellular, extracellular and total virus titres were determined. The viral replication in cell cultures was confirmed by PCR. Most sensitive to the virus were the primary and permanent cell cultures of sheep and rabbit origin, the least sensitive were the permanent cell cultures. Over the permanent cell cultures monolayer changes appeared on 3rd–4th day, then increased, and between the 5th and 7th days covered most space until the 7th–12th day when complete loss of monolayer was observed. Viral yield of EBTR and MDBK varied from log₁₀^{3.33} to log₁₀^{3.76} TCID₅₀/mL. Cytopathic effect after stationary culturing was found by hour 12–24: later then the roller cultivation and the viral titres were by 0.5 to 1.0 log₁₀ lower. DNA from 045 gene, 392 bp of size, was amplified successfully by PCR. The primary and permanent cell cultures of sheep origin were the most suitable for cultivation of Orf viruses, because the viruses were multiplied in highest quantity. Upon use of primary and permanent cell cultures of sheep origin, the cytopathic effect was the strongest and depended on the type of cultivation: roller or stationary. The used PCR variant was able to confirm the Orf virus in samples as well as in cell cultures.

Key words: cell culture, Orf virus, PCR

INTRODUCTION

The causal agent of ecthyma contagiosum is the Orf virus from the genus *Parapoxvirus* of family Poxviridae. The infection is often spread among small ru-

minants – sheep and goats in the countries with developed sheep farming, causing pustular rashes on the lips, genital area, legs and udder. In newborn and growing

sheep and goats, the virus causes pustular and papular rashes around the mouth, inability for suckling from their mothers, resulting in growth retardation or death. Contagious ecthyma is a zoonotic skin disease which affects the hands of people in contact with infected sheep (Paiba *et al.* 1999). The infection can cause lymphadenitis, generalised malaise or bullous pemphigoid (Leavell *et al.*, 1968; Wilkinson, 1977; Murphy & Ralphs, 1996).

The Orf virus is cultivated successfully in primary cell cultures derived from embryonic sheep skin (Greig, 1957) and sheep testis (Plowright *et al.*, 1959; Said 2013), rabbit kidney (Kuyumdziev & Todorov, 1962). Using primary cell cultures from bovine testis cells, Balassu & Robinson (1987) have ascertained new DNA replication 4–6 hours post infection and replication plateau between the 25th and 30th h. Accumulation of polypeptides is observed 10 h after inoculation with peak between post infection hours 14 and 16, with up to 35 polypeptides. Infectious virus has been ascertained between hours 16 and 18, which were produced until post inoculation hour 40.

Attempts for adaptation of Orf virus on the different primary cell cultures – lamb testicle (Lt), lamb kidney (Lk), calf testicle (Ct), calf kidney (Ck) and permanent cell cultures from monkey kidney (MK – Vero), swine kidney (SK), rabbit kidney (RK), diploid fibroblasts, HELA, BHK 21, HEP have been performed (Traykova, 1982). The cited author succeeded to cultivate the virus in primary cell of sheep and bovine origin and to reproduce the disease with cell culture virus on lambs. In MK permanent cell cultures, the cytopathic effect was observed after 2–3 passages and disappeared after the fifth passage. Howsawi & Abu Elsein (2000) infected primary sheep tes-

tis cell cultures and adapted the Orf virus on Vero cells. The development of the virus was demonstrated by neutralisation with positive serum.

For development of diagnostic methods and immunoprophylaxis, it is necessary to detect the most suitable cell line, providing accumulation of sufficient quantity of virus. This is accompanied with difficulties, because different virus isolates have different ability to adapt to the cell cultures. The purpose of the current study was to study the cultural characteristics of Orf virus, isolated from sheep and goats in Bulgaria.

MATERIALS AND METHODS

Cell cultures

Different primary and permanent cell cultures were used for determination of sensitivity for Orf virus reproduction. Primary cell cultures – sheep testis (ST), sheep kidney (SK) and rabbit kidney cells (RK) and permanent cell lines – foetal sheep kidney (FSK), sheep foetal thymus (SFT), bovine trachea (BTR), embryonic bovine trachea (EBTR), Madin Darby bovine kidney (MDBK) and baby hamster kidney (BHK 21) were used.

As growing media MEM Eagle with Hank's or Earle salts, Media 199 with addition of antibiotics (penicillin 100 UI/mL, streptomycin 100 µg/mL), 0.2 M L-glutamine, non-essential amino acids, 0.075% sodium bicarbonate and 10% foetal bovine serum (FBS) were used. The same media and additives were used as maintenance medium, but at lower quantity – 2% FBS. Inoculation of cell cultures was performed with and without adsorption for 2 h at 37 °C, or by direct dispensing of inocula into the growth media. Multiplications of the viruses were controlled daily for presence of cytopathic effect

over the monolayer. During the attempts for primary isolation, cell cultures were tracked until the 15th day post inoculation and viral development was evaluated microscopically. In the presence of cytopathic changes over the monolayer, the viruses were cultivated on the same cell culture for 3 and more consecutive passages. Non-inoculated cell cultures were used as controls, and evaluated in parallel to inoculated ones.

Dynamics of Orf virus multiplication was traced in more than five cell cultures after performing 10 consecutive passages. Cytopathic effect strength was evaluated as 1+ when 25% of the monolayer was affected and 4+ (100% affected monolayer). The serial passages were titrated additionally for determination of most suitable cell cultures for virus growth.

Two types of viral cultivations: stationary and roller were used for all Orf virus isolates on ST primary cell culture.

For determination of intracellular, extracellular and total Orf virus titres, cell cultures (ST, RK, SFT) were inoculated with 0.2 mL viral suspension containing 0.01 TCID₅₀/mL. After two-hour adsorption the inoculum was poured out, the infected monolayer was rinsed and was dispensed onto maintenance media. At defined intervals after inoculation (24, 48, 72, 96, and 120th h) two tubes with infected cell cultures were taken out and the titres of the intracellular, extracellular and total virus counts were determined. Viral titres in TCID₅₀/mL were calculated as per Reed & Muench (1938).

Viral samples

Crusts or nasal swabs from 14 infected sheep and 16 goats with signs of ecthyma contagiosum from Pasarel, Troyan, Kyustendil, Bankya, Rakitnitsa, Stara Zagora and Avren were used for cell cultures inoculation. Additionally crusts from two tars from a national hunting farm in Smolyan region were investigated. The Phylaxia vaccinal strain was used as reference. Sterile minced crusts were used for preparation of 10% suspensions in a mortar with quartz sand and saline or phosphate buffered saline (PBS) with pH 7.2. Antibiotics – penicillin 500 UI/mL and streptomycin 500 µg/mL were added to the suspensions. Samples were centrifuged at 4,000 rpm, 15 min and the supernatants were used for inoculation of cell cultures.

Polymerase chain reaction for confirmation of virus presence

Field samples from sheep and goats with signs of ecthyma contagiosum were examined by polymerase chain reaction (PCR) for demonstration of 045 gene. Viral DNA was extracted by DNA genomic kit (Qiagen, Germany) according to the manufacturer's instruction. Presence of DNA was checked after electrophoresis in agarose gel, and the quantity of viral DNA was determined by spectrophotometer Gene Quant II. Primers, multiplying the highly conservative gene 045, encoding the late transcription factor VLTF-1 of Orf virus and the parameters described by Kottaridi *et al.*, (2006) were used for performing the PCR (Table 1). As a posi-

Table 1. Nucleotide sequencing of used primers and the size of obtained products

Primers	Nucleotide sequencing	Product size, bp
Forward	5'-CCTACTTCTCGGAGTTCAGC-3'	392
Reverse	5'-GCAGCACTTCTCCTCGTAG-3'	392

Table 2. Data for cell cultures inoculated with the Pasarel isolate – time for appearance of the cytopathic effect, its strength and final cell culture titres

Cell cultures	Strength of cytopathic effect after inoculation												Titre, TCID ₅₀ /mL
	Days												
	1	2	3	4	5	6	7	8	9	10	11	12	
ST	–	–	1+	2+	3+	3+	3+	4+	4+	4+	4+	4+	10 ⁴
SK	–	–	–	1+	1+	2+	2+	3+	3+	3+	4+	4+	10 ^{2.76}
RK	–	–	–	1+	2+	2+	3+	3+	4+	4+	4+	4+	10 ^{3.73}
MDBK	–	–	1+	1+	1+	2+	2+	3+	3+	3+	4+	4+	10 ^{3.76}
BTR	–	–	–	–	–	1+	1+	2+	2+	3+	3+	4+	10 ^{2.33}
EBTR	–	–	–	–	1+	1+	2+	2+	3+	3+	4+	4+	10 ^{3.33}
FSK	–	–	–	1+	2+	2+	3+	3+	3+	4+	4+	4+	10 ^{3.5}
BHK 21	–	–	–	1–2+	2+	2+	3+	3+	4+	4+	4+	4+	10 ^{2.76}
SFT	–	–	–	1+	2+	2+	3+	4+	4+	4+	4+	4+	10 ^{4.33}

Note: 1+ to 4+: strength of cytopathic effects. ST – sheep testis, SK – sheep kidney, RK – rabbit kidney, MDBK – Madin Darbi bovine kidney, BTR – bovine trachea, EBTR – embryonal bovine trachea, FSK – foetal sheep kidney, BHK 21 – baby hamster kidney, SFT – sheep foetal thymus.

tive control Phylaxia vaccinal strain was used. In the same reaction six Orf isolates, adapted on permanent SFT cell cultures were investigated.

RESULTS

From performed studies, we ascertained that the primary and permanent cell cultures of sheep and rabbit origin (ST, SK and RK) were the most sensitive to Orf virus while permanent cell cultures (MDBK, BTr, EBTr, BHK-21 and SFT) were less sensitive (Table 2).

When primary cell cultures SK and ST were used, Orf virus isolates were cultivated for more than 10 passages. Appearance and strength of the cytopathic effect depended on infectious viral doses, used for inoculation and on the number of

passages. Until 4–5 passages the cytopathic effect appeared by the 3rd–4th day and was characterised by rounding and ballooning of cells. The monolayer of the cells in the first few days post infection was intact, and on 5th–6th day post inoculation the cytopathic effect was strengthened, as part of the cells was dropped off and foci with single rounded cells on their periphery were formed. After 4–5 passages the cytopathic effect was more intensive, and appeared earlier (Fig.1B), covering the entire monolayer (Fig.1C). Multiple foci of damaged cells were visible (Fig. 1D). The monolayer was torn and most of the cells were dropped off into the media. After the 10th passage, the cytopathic effect appeared earlier at post inoculation hour 48. In control uninfected

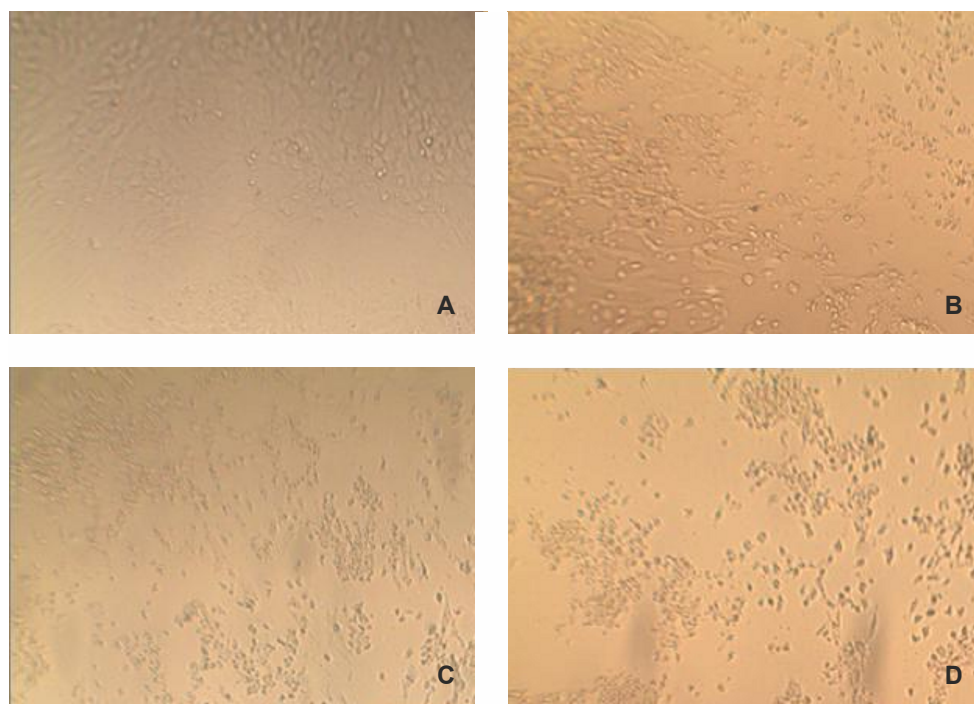


Fig. 1. Cytopathic effect of Pasarel viral isolate on 5th passage on primary cell culture of sheep testis. A) non-inoculated control cell culture; B) cytopathic effect by the 3rd day; C) cytopathic effect by the 6th day; D) total cellular monolayer destruction on day 8-9 (magnification $\times 200$).

Table 3. Data for sensitivity of primary and permanent cell cultures for growing of the Pasarel field strain for 10 consecutive passages

Cell cultures	Infectious titres in consecutive passages, TCID ₅₀ /mL				
	1 passage	3 passage	5 passage	8 passage	10 passage
ST	$10^{2.73}$	$10^{3.73}$	$10^{4.5}$	$10^{4.73}$	$10^{4.73}$
SK	$10^{1.5}$	$10^{2.5}$	$10^{2.76}$	$10^{2.76}$	$10^{2.76}$
RK	$10^{2.33}$	$10^{3.33}$	$10^{3.73}$	$10^{3.73}$	$10^{3.73}$
SFT	$10^{2.50}$	$10^{3.66}$	$10^{3.50}$	$10^{4.0}$	$10^{4.33}$
FSK	$10^{2.73}$	$10^{2.33}$	$10^{3.50}$	$10^{3.66}$	$10^{3.73}$

Primary cell cultures: ST – sheep testis, SK – sheep kidney and RK – rabbit kidney. Permanent cell lines: SFT – sheep foetal thymus and FSK – foetal sheep kidney.

cell cultures, changes of monolayer were not visible (Fig. 1A).

After inoculation with low infectious doses of the virus, complete dropping of cells from the monolayer was observed

4–5 days after the infection, but after using the high viral dose the monolayer dropped off in the medium earlier: after 2–3 days.

Table 4. Data for intracellular, extracellular and total virus titres from investigated Orf isolates on SFT permanent cell culture

Viral isolate	Viral titre, TCID ₅₀ /mL		
	intracellular	extracellular	total
Phylaxia reference	10 ^{2.0}	10 ^{3.0}	10 ^{3.66}
Pasarel – goat	10 ^{2.66}	10 ^{3.66}	10 ^{4.33}
Tar 1	10 ^{2.0}	10 ^{3.0}	10 ^{3.50}
Troyan – goat	10 ^{2.0}	10 ^{3.0}	10 ^{4.00}
Kyustendil – sheep	10 ^{3.0}	10 ^{3.0}	10 ^{4.50}

Upon titration of the virus on ST cell culture, the extra cellular virus titre was 10^{3.66} TCID₅₀ /mL, the intracellular one – 10^{2.66} TCID₅₀/mL and the total virus titre was 10^{4.0} TCID₅₀/mL. After using SK cell culture, the cytopathic effect appeared later with lower titres (by 0.5 to 1.5 log10) compared to the ST cell culture.

In using RK cell cultures, the cytopathic effect appeared by the 2nd–3rd day, as cells were rounded and lost connection with neighbouring cells. After appearance of the cytopathic effect, after 4-5 days almost all cells were affected, a great part of them dropped off to the maintenance media. The viral titres ranged from log 10^{2.33} to log 10^{3.66} TCID₅₀/mL.

By using permanent cell cultures of sheep (SFT, SFK) and bovine origin (BTR, EBTR and MDBK) the changes of monolayer appeared by the 3rd–4th day, and after 5 to 7 days they covered a larger area of cell monolayer. In SFT and bovine cell cultures, dropping off of a great part of monolayer cells into the maintenance media was observed by 7th–9th and 10th–12th day, respectively. Viral titres on EBTR and MDBK cell cultures ranged from log10^{3.33} to log10^{3.76} TCID₅₀/mL.

The investigation of the dynamics of multiplication of Pasarel strain on primary and permanent cell cultures for 10 consecutive passages, the viral growth initially increased up to the 5th passage. After

that, in permanent cell cultures of sheep and primary cell cultures of rabbit origin, a constant level of infectious titres was ascertained (Table 3).

The cytopathic effect in primary ST cell cultures of investigated Orf virus isolates in roller cultivation appeared earlier (after the 12th h), compared to the stationary cultivation (after the 24th h). Depending on cell cultures (sheep or bovine) the viral titres were by 0.5 to 1.0 log10 lower vs stationary cultivation.

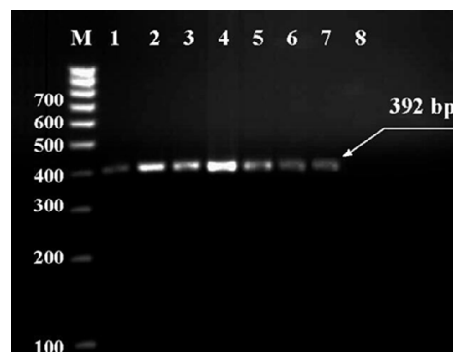


Fig. 2. Amplified products of 045 gene, produced by PCR of six Orf isolates, adapted on SFT permanent cell culture. The arrow designates 392 bp product. Lane M: marker of molecular weights 100 bp, lane 1: Phylaxia reference strain; lane 2: Pasarel goat isolate; lane 3: Troyan goat isolate; lane 4: Kyustendil sheep isolate; lane 5: Tar 1 isolate; lane 6: Avren sheep isolate; lane 7: crusts suspension from Avren isolate before cultivation on cell culture; lane 8: non-inoculated control SFT cell culture.

After determination of intracellular, extracellular and total virus counts of five viral Orf isolates, the quantity of the intracellular virus was the lowest, while the quantity of extracellular and total viruses were the highest when permanent SFT cell culture was used (Table 4).

The PCR used primers, multiplying a fragment from the highly conservative 045 gene of the Orf virus, encoding the late transcription factor VLTF-1. With the used primers and parameters of reaction, DNA of 045 gene (size 392 bp) was amplified from the six tested isolates and from the Phylaxia control reference strain (Fig. 2).

DISCUSSION

For studies of the Orf virus cultural characteristics, we used primary and permanent cell cultures of different origin. Studies on the Orf virus replication were performed by Balassu & Robinson (1987) on primary cell cultures. The authors studied the kinetics of accumulation of the viral DNA, analysed the viral polypeptides and the production of infectious virus. They have found that the virus yield on the primary cell cultures from sheep testicles and rabbit kidneys were the highest and have concluded that they were the most suitable cells for multiplication of the virus, which was confirmed by the current investigation. Most probably the receptors on this type of cells were most suitable for attachment and growth of the virus.

With increasing the number of passages, the titres of tested isolates also increased, demonstrating the adaptation of the agents to the cells with increasing the number of passages. The obtained data are similar to the results of Greig (1957) for primary cell cultures from sheep embryonic skin after 10 serial passages. The

observed cytopathic effect is characterised by rounding of many cells, followed by increased granularity of the cellular cytoplasm and fragmentation. Most of the cells affected by the Orf virus are detached from the walls and floated in the maintenance medium with advancing of the viral development. Most of the cells that remained on the walls were accumulated as plaques with long cytoplasmic shoots. With increasing the number of passages, the cytopathic effect appeared earlier. Houssawi & Abu Elsein (2000) have made two passages of Orf virus on primary sheep testicles and after that the virus was adapted on permanent Vero CC. Pyknosis and cell dropping off at 5–8 days post inoculation have been observed by Said (2013). After infection of chicken embryo with positive cell culture isolates, over the chorioallantoic membrane the author found thickening of the membrane in nine samples and seven of them were confirmed as Orf viruses by agar gel immune diffusion test.

In the present study the titres of virus cultivated in the permanent cell cultures of bovine origin were lower, but these cell cultures may also be used for cultivation. Most probably the reason for lower titres was the incomplete correspondence between cellular and viral receptors. Viral multiplication after the 3rd subcultivation after using the SPEV cell line was not ascertained. This indicates discrepancy of viral and cellular receptors, which did not allow optimal viral development resulting in progressive decrease in the number of the viral infectious particles. Another probable reason is the formation of incomplete or defective viral particles that interfere with the infectious virus. A lot of experiments for Orf virus cultivation on the primary or permanent cell cultures have not been successful. Using a wide

variety of permanent cell cultures, Traykova (1982) found cytopathic effect only in MK cell cultures, that appeared on 4th–5th day post inoculation and resulted in rounding and ballooning of the cells, without foci formation. Cytopathic effect was observed only on 2nd and 3rd passage and in 4–5th passage it was completely lost and the culture stayed unchanged. In other permanent cell cultures, the titres were lower, in permanent RK the cytopathic effect disappeared after the second passage, while in SPEV – after the third one.

Nagington & Whittle (1961) have inoculated amniotic cells with material from man with contagious ecthyma and 18 h post infection they found rounding, scattering and granulation of the cells and absence of other changes till the 15th day. After that they have observed few empty spaces on the edge of the monolayer and have attempted to cultivate the virus in permanent cell cultures. In HeLa, MK 2, Am9 cell cultures they did not observe development of the virus for 5 passages.

In the present study Orf viruses, initially adapted on the primary cell cultures from ST were used on permanent cell cultures of sheep and bovine origin. Cultivation on the permanent cell cultures of sheep origin demonstrated the adaptation of the virus on this type of cell cultures. On cell cultures of bovine origin clear cytopathic effect, which later weakened was observed after the first 2–3 passages. Sheep parapox virus was cultivated and was titred by plaque method on the permanent MDBK cell culture by Kruse & Weber (2001), and was used for studying the apoptosis. The authors proved selective induction of apoptosis in antigen-presenting cells of mice by sheep parapoxvirus.

The used PCR variant successfully multiplied part of DNA both from field

samples as well as from viral isolates adapted on cell culture. This is an evidence that isolates obtained and adapted on cell cultures were Orf viruses and confirmed their successful cultivation.

CONCLUSIONS

Primary and permanent cell cultures of sheep origin were found as most suitable for cultivation of Orf viruses because the viruses were multiplied with highest quantity.

The cytopathic effect was the strongest in primary and permanent cell cultures of sheep origin and depended on the type of cultivation: roller or stationary.

The used variant of PCR was capable to confirm the Orf virus in samples and in cell cultures.

REFERENCES

- Balassu, T. C. & A. J. Robinson, 1987. Orf virus replication in bovine testis cells: kinetics of viral DNA, polypeptide, and infectious virus production and analysis of virion polypeptides. *Archives of Virology*, **97**, 267–281.
- Greig, A., 1957. Contagious ecthyma of sheep. II - *In vitro* cultivation of the virus, *Canadian Journal of Comparative Medicine*, **21**, 304–308.
- Housawi, F. & E. Abu-Elzein, 2000. Contagious ecthyma associated with myiasis in sheep. *Revue scientifique et technique (International Office of Epizootics)*, **19**, 863–866.
- Kottaridi, C., K. Nomikou, R. Lelli, P. Markoulatos & O. Mangana, 2006. Laboratory diagnosis of contagious ecthyma: Comparison of different PCR protocols with virus isolation in cell culture. *Journal of Virological Methods*, **134**, 119–124.
- Kruse, N. & O. Weber, 2001. Selective induction of apoptosis in antigen presenting

Studies on cultural characteristics of contagious ecthyma (Orf) virus

- cells in mice by parapoxvirus ovis. *Journal of Virological Methods*, **75**, 4699–4704.
- Kuyumdziev, I. & T. Todorov, 1962. Tissue cultural and electronic microscopy studies on virus of Contagious Ecthyma. *Microbiology Institute* **13**, 21–28.
- Leavell, U. W., M. J. McNamara, G. R. Muelin, W. M. Talbert, R. C. Rucker & A. J. Dalton, 1968. Orf. Report of 19 human cases with clinical and pathological observations. *Journal of the American Veterinary Medical Association*, **204**, 109–116.
- Murphy, J. K. & I. G. Ralphs, 1996. Bullous pemphigoid complicating human orf. *British Journal of Dermatology*, **134**, 929–930.
- Nagington, J. & C. Whittle, 1961. Human orf. Isolation of the virus by tissue culture. *British Medical Journal*, **18**, 1324–1327.
- Paiba, G. A. D., R. H. Thomas, K. L. Morgan, M. Bennett, R. L. Salmon, R. Chalmers, S. M. Kench, T. J. Coleman, D. Meadows, P. Morgan-Capner, P. Softley & M. Sillis, 1999. Orf (contagious pustular dermatitis) in farmworkers: Prevalence and risk factors in three areas of England. *The Veterinary Record*, **145**, 7–11.
- Plowright, W., M. A. Witcomb & R. D. Ferris, 1959. Studies with a strain of contagious pustular dermatitis virus in tissue culture. *Archives für die gesamte Virusforschung*, **9**, 214–231.
- Reed, L. J. & H. Muench, 1938. A simple method of estimating fifty percent endpoints. *American Journal of Hygiene*, **27**, 493–497.
- Said, A., 2013. Trials for isolation of contagious pustular dermatitis virus (CPDV) from sheep in Ismailia Governorate. *Research in Zoology*, **3**, 10–14.
- Traykova, M., 1982. Attempts for adapting virus of contagious ecthyma on cell cultures. *Veterinary Medicine Sciences (Sofia)*, **10**, 18–25 (BG).
- Wilkinson, J. D., 1977. Orf: A family with unusual complications. *British Journal of Dermatology*, **97**, 447–450.

Paper received 12.06.2015; accepted for publication 13.11.2015

Correspondence:

Lyudmil Ivanov
National Diagnostic and Research Veterinary
Medical Institute
15 P. Slaveikov Blvd
1000, Sofia,
tel. +359 889 95 96 95
e-mail: tse_sz@abv.bg