VETERINARSKI ARHIV 82 (5), 495-504, 2012

Fluorescent *in-situ* hybridization technique for the detection and localization of classical swine fever virus in infected tissues

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NAGARAJAN, K., G. SAIKUMAR: Fluorescent *in-situ* hybridization technique for the detection and localization of classical swine fever virus in infected tissues. Vet. arhiv 82, 495-504, 2012.

ABSTRACT

Classical swine fever (CSF) may be diagnosed by various conventional techniques but nucleic acid based methods have gained popularity because of their high sensitivity and specificity. For detection of viral RNA in infected tissues, a fluorescent *in-situ* hybridization (FISH) based method was developed using a 308 bp biotinylated DNA probe targeting E2/NS2 gene of CSFV. *In-situ* RNA/DNA hybrids were detected with fluorescently labeled Streptavidin conjugate and viewed under a fluorescent microscope. Viral nucleic acids could be demonstrated in the lymphoid tissues such as the spleen and lymph nodes collected from CSFV infected animals. Infected macrophages and reticuloendothelial cells revealed bright intracytoplasmic fluorescence. In conclusion, the fluorescent in situ hybridization technique used in the study provided an effective means of detection and localization of CSFV nucleic acids.

Key words: classical swine fever virus, fluorescent *in-situ* hybridization, virus localization

Introduction

Classical swine fever (CSF) is an important, highly contagious disease of pigs caused by the classical swine fever virus (CSFV), a member of the genus *Pestivirus* of the *Flaviviridae* family, which also includes bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) (WENGLER et al., 1995). CSFV is an enveloped, positive-sensed, single stranded RNA virus, which is spherical in shape and 40-60 nm in size, with a genome of approximately 12.3 kb (MOORMANN et al., 1990; HA et al., 2004). The disease

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ISSN 0372-5480
Printed in Croatia

is characterized by a per-acute, acute, sub-acute, chronic, atypical or unapparent course (ROBERTSON and OWEN, 1994) depending on the virulence and dose of the virus and the age and breed of the pigs, besides other host and environmental factors (MOENNIG et al., 2003). CSF is conventionally diagnosed on the basis of clinical signs and necropsy lesions. However, the high degree of variability in the clinical picture and the resemblance of the clinical syndrome to many other diseases precludes reliable clinical diagnosis. Confirmation of the presumptive diagnosis needs to be done by standard laboratory tests (PEARSON, 1992). Improved and emerging diagnostics for CSF include viral antigen/ genome based detection methods such as Immunohistochemistry, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and *in-situ* Hybridization (ISH) (LIU et al., 1991; CHOI and CHAE, 2003; HANDEL et al., 2004). Fluorescent *in-situ* Hybridization (FISH) is a valuable adjunct to such techniques for the detection of the viral genome in tissues and cells. The major advantage is the ability to determine the target cells containing the viral RNA. The purpose of the present study was to apply FISH for the detection of CSFV nucleic acid in formalin-fixed paraffin-embedded tissues from piglets experimentally infected with CSFV, and to demonstrate the diagnostic value of the method and its applicability in studies of viral pathogenesis.

Materials and methods

Animal inoculation. Eight numbers of white crossbred piglets of both sexes weighing around 20-22 kg, free from neutralizing antibodies against classical swine fever virus, were employed in the present study. Six piglets (No.1-6) were inoculated intranasally with a $10^{4.0}$ tissue culture infective dose 50% ($TCID_{50}$) of CSFV (INB03) isolate and two mock infected piglets were maintained as controls. Following inoculation, clinical observations in respect of general condition, rectal temperature and other clinical signs were made during the experiment.

Clinical observation and collection of tissue samples. Following inoculation, the daily rectal temperatures were recorded until death or termination. Clinical observations were made throughout the course of the experiment. Immediately after death or sacrifice (7, 14, 21 days post infection (DPI) as per experimental schedule), representative lymphoid tissues, such as: spleen, cervical and mesenteric lymph nodes, tonsil, and ileocaecal junction, were collected on ice and frozen at -70 ºC in 10% buffered neutral formalin and fixed for 48 h at room temperature. The tissues were subjected to routine histological processing and 6 µm thin sections were mounted on 3-aminopropyltriethoxysilane (Sigma-Aldrich, USA) coated slides and stored at 4 °C under dry conditions for use in the FISH method.

RNA extraction. Standard procedures were used to avoid false-positive results due to contamination (LIU et al., 2011). These included the use of separate rooms for the

preparation of master mixes, extraction of RNA, and addition of RNA to the reaction tubes. Total RNA was extracted from all the tissues collected at various intervals, using TRIzol® Reagent (Life Technologies-Invitrogen, USA), as per the manufacturer's recommendation.

Primers. The forward and reverse primers (HCVgp5) 5'-ATATATGCTCAAGGGCGAGT-3' (nucleotides 3378-3397) and (HCVgp8) 5'-ACAGCAGTAGTATCCATTTCTTTA-3' (nucleotides 3662-3685), respectively (KATZ et al., 1993) were used in this study.

Reverse transcription. First-strand cDNA synthesis was performed in a 20 µL reaction volume. 5 ng of total RNA was mixed with 10 pmol specific reverse primers (HCVgp8), heated to 65 ºC for 5 min and quick chilled on ice. Then, 4 µL of 5X first-strand buffer (Sigma-Aldrich, USA), 1 µL 10 mM dNTP mix (Sigma-Aldrich, USA), 2 µL of 0.1M DTT, 40 units of Recombinant ribonuclease inhibitor (RNAse-out, Invitrogen, USA) and nuclease free water, to adjust the volume, were added. The contents were mixed and incubated at 37 ºC for 2 min, followed by the addition of 200 units of M-MLV RT (Sigma-Aldrich, USA). The tube was incubated at 37 ºC for 50 min and heat inactivated 70 °C for 15 min.

Polymerase chain reaction. PCR was carried out in a 50 µL reaction volume consisting of 2 µL of first- strand cDNA, 5 µL of 10X PCR buffer (Invitrogen, USA), 10 pmol each specific forward and reverse primers, 1.25 µL of 50 mM MgCl2 (Invitrogen, USA), 2 µL of 2.5 mM dNTP mix (Invitrogen, USA), 0.2 µL of Taq DNA Polymerase (5 units/µL) (Invitrogen, USA) and nuclease free water to make up the volume. The reaction was carried out in a thermocycler (Techne Genius, UK) using the following thermal profile and cycling conditions: initial denaturation for 3 min at 94 ºC followed by 35 cycles of denaturation for 45 s at 94 ºC, annealing for 45 s at 60 ºC, elongation for 45 s at 72 ºC and final elongation for 10 min at 72 ºC. RT-PCR amplicons were analyzed by electrophoresis on 1% agarose gel as per standard procedures. The RT-PCR product was purified using a STRATAPREP PCR product purification kit (Stratagene, USA), as per the manufacturer's protocol.

Generation of cDNA probe. The 308 bp RT-PCR amplicon from the E2/NS2 genomic region of the INB/03 strain of CSFV was cloned into the PCR-4 Topo® vector using a Topo-TA cloning® kit (Invitrogen, USA) for recombinant plasmid preparation, as per the manufacturer's recommended procedure, to generate a biotinylated cDNA probe. The labeling reaction mixture consisted of 5 µL of 10X PCR buffer without $MgCl_2$ (Invitrogen, USA), 2.5 µL of 25 mM $MgCl_2$ (Invitrogen, USA), 2.5 µL of labeling dNTP mix without biotin-14-dATP, 4.3 µL of 0.4 mM biotin-14 dATP (Invitrogen, USA), 5 pmol each of forward and reverse primers, 0.5 µL of 0.05 µg/µL recombinant plasmid DNA, 1.0 µL of 5 units/µL Taq DNA Polymerase (Invitrogen, USA) and nuclease free water to make up volume to 50 µL. The reaction was placed in a thermocycler, and the

cycling conditions were as follows: initial denaturation for 7 min at 94 ºC followed by 35 cycles of denaturation for 45 s at 94 ºC, annealing for 45 s at 60 ºC, elongation for 45 s at 72 ºC and final elongation for 15 minutes at 72 ºC. The purification of the probe was done using a STRATAPREP PCR product purification kit (Stratagene, USA) as per the manufacturer's recommended protocol.

Quantitation of biotinylated cDNA probes. The biotinylated cDNA probes were electrophoresed on a 0.8% Agarose-TBE gel containing 0.5 µg/ mL ethidium bromide along with a 100-bp quantitative DNA molecular weight marker (Life technologies, USA). The respective sizes of the biotinylated probes were determined by comparison with 100 bp DNA molecular weight markers, whereas quantitation was effected by comparing the band intensities of the probes with those of the quantitative DNA molecular weight marker.

Fluorescent in-situ hybridization. Preparation of tissue sections. The tissue sections were incubated at 60 ºC for 30 min followed by deparaffinization in xylene (2 × 10 min) and rehydration in a graded ethanol series (100%, 95%, 70% and 50% for 5 min each). Subsequently the sections were washed with diethyl pyrocarbonate (DEPC) treated water (2 × 5 min), DEPC treated phosphate buffered saline (PBS) (pH 7.4) (2 × 5 min), DEPC treated PBS containing 100 mM glycine (2 × 5 min), DEPC treated PBS with 0.3% triton X-100 (1 × 15 min), DEPC treated PBS (2 × 5 min). In order to increase the target accessibility and probe penetration, tissue sections were digested with 200µL of 40 µg/ mL proteinase-K solution for 45 min at 37 ºC in a hybridization chamber (HYBAID, U.K). Subsequently the sections were post fixed in 4% buffered paraformaldehyde (pH 7.4) for 15 minutes at 4 ºC and washed in DEPC treated PBS (2 × 5 min).

Pre-hybridization, hybridization and post- hybridization washing. Hybridization was carried out in a plastic container with a tight fitting lid. Blotting paper, moistened with 6X sodium chloride and sodium citrate solution (SSC), was placed at the bottom of the dish. A raised platform was prepared by fixing glass rods in order to avoid contact of the slides with the moist bottom. Care was taken to ensure that the slides kept over the glass rods were on a level plane. The sections were prehybridized with 200 µL of pre-hybridization buffer (4X SSC containing 50% (v/v) deionized formamide) under siliconized coverslips at 42 ºC for 1h. The fluid was drained and slides rinsed in 2X SSC for 1 min. Finally the sections were overlaid with 70 µL of hybridization buffer (40% deionized formamide containing 4X SSC, 1X Denhardt's reagent, 10% Dextran sulphate, 10 mM DTT, 2 mg/ mL denatured salmon sperm DNA and heat denatured biotin labeled probe 100 ng/µL) The sections were covered with siliconized coverslips and sealed with rubber cement. Denaturation was carried out at 70 ºC on a heated platform for 5 min, followed by quenching on an ice-cold platform for 3 min. The slides were transferred to the hybridization chamber and incubated overnight at 42 ºC. Post hybridization washing

steps included rinsing in 2X SSC (1 × 10 min), 2X SSC (2 × 15 min, 37 ºC), 1X SSC (2 × 15 min, 37 ºC) and 0.1X SSC (2 × 30 min, 42 ºC).

Detection. Tissue sections were washed in TNT buffer (100 mM Tris-HCl, 150mM NaCl, 0.05% Tween-20) pH 7.5 twice at RT and incubated with 200 µL of blocking buffer (TBS containing 3% BSA and 0.1% TritonX-100) for 1 hr at 37 ºC. The blocking buffer was then decanted and the sections were incubated with fluorescently labeled streptavidin conjugate (Bangalore Genei, India) at a dilution of 1:250 for 30 minutes at 37 ºC. The sections were then washed with TNT buffer (3 × 10 min). Slides were prepared for viewing under a fluorescent microscope by dehydration in graded ethanol series and mounting in 50% buffered glycerol gelatin. Positive signals were identified by the presence of fluorescence. A CSFV negative control, probe control, conjugates and substrate controls were included in every batch.

Results

Piglets experimentally infected with CSFV (INB/03) revealed typical acute clinical signs of CSF. Within 6 to 11 DPI, all inoculated pigs had either died or were sacrificed *in extremis*. The control piglets remained clinically normal. FISH was performed on the lymphoid organs, which included the tonsils, mesenteric and cervical lymph nodes, spleen, and ileocaecal junctions collected at various intervals of the experiment. Table 1 summarizes the results of FISH. Tonsils of all the infected piglets revealed fluorescence of very high intensity, especially in piglet no. 5 and 6, which died on 7 and 6 DPI, respectively. Signals were observed mainly within the tonsillar crypt epithelial cells, follicular areas, and occasionally in the germinal centers. Similarly, fluorescence of high to moderate intensity was observed in other lymphoid organs, such as the lymph nodes and spleen, particularly in the center of depleted follicles and within mononuclear cells of the cell poor substance. Positive signals were also detected in the macrophages and reticuloendothelial cells as a thin rim of bright fluorescence intracytoplasmically, peripheral to the nuclei (Fig. 1 and Fig. 2). Only one animal (No.3), which was sacrificed on 11 DPI, revealed appreciable fluorescence, especially in the ileocaecal junction. These signals were detected within the depleted follicles of the Peyer's patches having severe histological lesions of follicular degeneration and necrosis. CSFV negative tissue controls, probe control, conjugate and substrate controls were found clear and free of fluorescence for CSFV genome.

Table 1. Results of FISH on tissue samples collected from piglets experimentally infected with CSFV (INB/03)

Tissue and organs collected	Details of piglets and result of fluorescent in-situ hybridization (FISH)							
	1 (Died, 9 PID)	2 (Died, 8 PID)	3 (Sacrificed, 11 PID)	4 (Died, 11 PID) autolysed	5 (Sacrificed, 7 PID)	6 (Died, 6 PID)	7 Control (Sacrificed, 21 PID)	8 Control (Sacrificed, 21 PID)
Tonsil	3+	3+	3+	ND	4+	4+	•	•
MLN	3+	3+	3+		3+	4+	•	•
CLN	3+	3+	3+		3+	4+	•	•
Spleen	3+	3+	2+		2+	2+	•	•
ICJ	•	•	1+		•	•	•	•

4+ = very high signal intensity, 3+ = high signal intensity, 2+ = moderate signal intensity, 1+ = low signal intensity, • = no signal, ND = not done

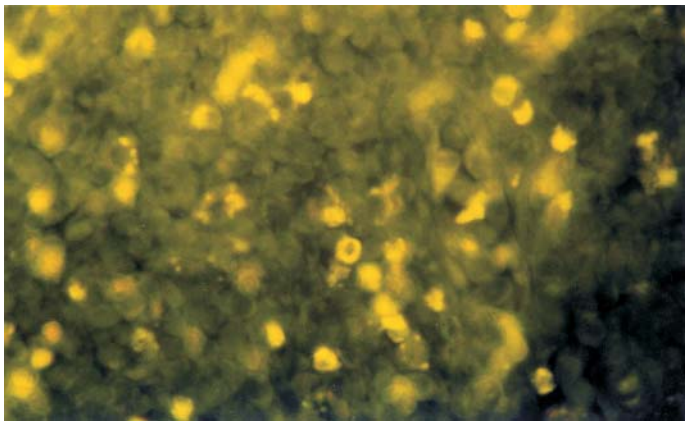

Fig. 1. Lymph node of the CSFV-INB/03 infected piglet revealing intracytoplasmic positive signals in the reticuloendothelial cells. FISH, Fluorescence, ×400.

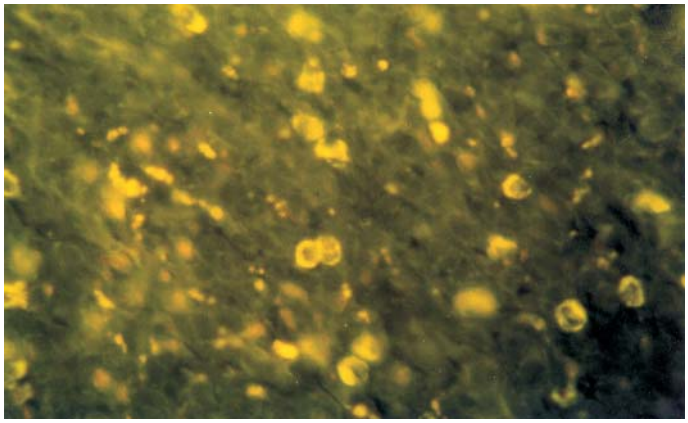

Fig. 2. Spleen of the CSFV-INB/03 infected piglet revealing intracytoplasmic positive signals in the reticuloendothelial cells. FISH, Fluorescence, ×400.

Discussion

The clinical signs, gross and histological lesions found in the infected piglets corroborated with observations that most CSFV infected animals die with widespread hemorrhage, thrombosis, splenic infarction, and encephalitis within 3 weeks of infection (NARITA et al., 2000). Lymphoid organs, which include tonsils, mesenteric and cervical lymph nodes, spleen, and ileocaecal junctions, collected at various intervals of the experiment, were tested for detection of CSFV nucleic acid by FISH. The tonsils of all the infected piglets revealed fluorescence of very high intensity. Similarly, fluorescence with high to moderate intensity was observed in other lymphoid organs such as the lymph nodes and spleen, particularly in the center of depleted follicles and within mononuclear cells of the cell poor substance (LIU et al., 2011). The pattern of signal distribution in the lymphoid organs, specifically in the germinal centers, which are rich in B cells and mononuclear phagocytes, was similar to the observation made earlier by SUSA et al. (1992), who reported high concentrations of virus localized in the lymphoid tissues. In contrast, CHOI and CHAE (2003) observed no hybridization signals within cells in the follicular centre. This difference may be attributed to the virulence of the virus and the nature of the infection, as their study was conducted on pigs with chronic infection. The distribution pattern of fluorescence observed in lymphoid tissues such as lymph nodes, spleen and Payer's patches, confirmed earlier findings by immunohistochemistry. In pigs acutely infected with the virulent strain Brescia, the CSFV antigen was detectable first in crypt tonsillar epithelial cells, follicular macrophages, reticular and endothelial cells in the spleen and lymph nodes, and the smooth muscle cells of the blood vessels

(SUMMERFIELD et al., 1998). Further, the distribution and the intensity of signals support earlier reports by histopathology and immunohistochemistry that the lymphoid tissues are the target organs (RESSANG, 1973; SANCHEZ-CORDEN et al., 2003). In conclusion, fluorescent *in-situ* hybridization with a biotinylated cDNA probe enabled detection and localization of the virus in the lymphoid organs. Like immunohistochemical methods, FISH enables direct visualization and localization of the virus in the infected tissues. Since most antibodies cannot distinguish between different related viruses, FISH can be used to study the pathogenic mechanisms of different pathotypes of CSFV. Since the target organs for CSFV are the lymphoid organs and the biotinylated cDNA probe works efficiently in these organs, the probe is suitable for diagnostic applications.

Acknowledgements

The authors would like to thank the Director of the I.V.R.I for providing the necessary facilities to carry out the work.

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Received: 11 June 2011
Accepted: 27 June 2012

NAGARAJAN, K., G. SAIKUMAR: Fluorescentna *in situ* hibridizacija za dokazivanje virusa klasične svinjske kuge i praćenje njegove lokalizacije u zaraženim tkivima. Vet. arhiv 82, 495-504, 2012.

SAŽETAK

Klasična svinjska kuga može se dijagnosticirati različitim uobičajenim metodama, ali se, zbog visoke osjetljivosti i specifičnosti, najčešće rabe metode zasnovane na dokazivanju specifične nukleinske kiseline. Za dokaz virusne RNA u zaraženom tkivu u ovom je radu razvijena fluorescentna *in situ* hibridizacija (FISH) uporabom biotinom obilježene DNA probe od 308 bp za ciljni gen E2/NS2 virusa klasične svinjske kuge. RNA/DNA hibridne molekule bile su dokazane uporabom konjugata obilježenog streptavidinom pretragom fluorescentnim mikroskopom. Virusna nukleinska kiselina bila je dokazana u limfoidnim tkivima odnosno u slezeni i limfnim čvorovima životinja zaraženih virusom. Fluorescencija je jasno bila izražena u citoplazmi zaraženih makrofaga i retikuloendotelijalnih

stanica. Može se zaključiti da fluorescentna *in situ* hibridizacija opisana u ovom radu pruža učinkovit alat za dokaz nukleinske kiseline virusa klasične svinjske kuge i njegove lokalizacije u zaraženim tkivima.

Ključne riječi: virus klasične svinjske kuge, fluorescentna *in-situ* hibridizacija, lokalizacija virusa