An Outbreak of Classical Swine Fever (CSF) in a Closed-Cycle Unit in Israel

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ABSTRACT

In a closed pig holding facility containing 425 sows, located in northern Israel, a Classical Swine Fever (CSF) outbreak occurred, which caused the loss of 267 sows (62.8% of the farm sow population), 4 boars (out of 6), and 828 growing/fattening pigs (41.4% of the total on the farm), through direct mortality, partial culling, and an unknown number of abortions. The CSF outbreak started among the pregnant sows, with high fever, cyanosis, abortions, lameness, vomiting, and mortality (24.8% of sows and 33.3% boars). At necropsy, hemorrhages were found on the parietal pleura, diaphragm, epicardium and kidney; lymph nodes were congested and hemorrhagic; necrotic lesions were present on the mucosa of the small intestine. CSF was diagnosed at Kimron Veterinary Institute by reverse transcriptase polymerase chain reaction (RT-PCR) method and with ELISA-antibody test on serum and ELISA-antigen test on organ homogenates. The results were confirmed by the EU Reference Laboratory at Hanover, Germany with Real Time–PCR on blood and organs. Phylogenetic analysis showed that CSFV sequences of the Israeli virus isolates belonged to genotype 2.1. Molecular typing and comparison of amino acids with CSFV subgroup 2.1 isolates from Southeast Asia and from Europe revealed that the CSFV isolate responsible for the Israeli outbreak was genetically most similar to a Chinese CSFV isolate. Involvement of wild boars, which were in contact with sows was suspected. Samples from blood and organs taken from wild boars found in the vicinity of the farm were found positive for CSF. Following confirmation of CSF, elimination of clinically sick animals and vaccination of the remaining population in the holding and the surrounding area were implemented.

Keywords: Classical Swine Fever, pig, wild-pig, hemorrhagic, abortion, molecular analysis, CSF genotype 2.1.

INTRODUCTION

Classical Swine Fever (CSF) is a highly contagious, multisystemic, hemorrhagic viral disease, induced by a single-stranded-RNA Pestivirus, belonging to the family *Flaviviridae*. CSF affects both domestic and wild pigs, of which the latter are considered to be the reservoir of this virus. Transmission occurs through the oral-nasal route, with first replication of virus at the tonsil level, followed by passage to regional lymph nodes followed by a viremia. Disease severity may range from mild to severe, and it may cause heavy losses in affected herds (1).

Clinical signs appearing as a result of CSF are fever, intense redness of the skin that can develop to cyanosis, lack of coordination of the hind legs, diarrhea, and pneumonia; abortions occur in pregnant animals (1).

Three clinical forms of CSF are recognized: acute, chron-

ic, and congenital or pre-natal (1). Differential diagnosis of CSF should consider African Swine Fever (ASF), Bovine Viral Diarrhea (BVD), Salmonellosis, acute Pasteurellosis, Erysipelotrix, Streptococcosis, and Leptospirosis. Other possibilities are poisoning by coumarin and anticoagulants which should also be taken into account.

The acute form is characterized by leucopenia, thrombocytopenia, diffused hemorrhagic petechiae and ecchymoses on skin and in lymph nodes, larynx, bladder, kidneys (diffused puntate lesions; so called "turkey egg kidney") and the ilio-caecal valve. Hemorrhagic multi-focal congestion of the spleen is typical but is not always present. Lymph nodes are enlarged and hemorrhagic. Lesions and perivascular cellular infiltrations are present in the central nervous system (CNS). The chronic form is characterized by necrotic-ulcerated lesions (so called "buttons") in the caecum and large intestine, generalized depletion of lymphoid tissues and inflammatory and hemorrhagic lesions may be present. The congenital form is characterized by microencephaly, hypoplasia of cerebellum and lungs, and dysmyelinogenesis of the CNS.

Diagnosis is based on examination of pathological and histopathological lesions, demonstration of viral RNA by RT-PCR, application of the immuno-fluorescence test (IFT) to cryostat sections, virus isolation and demonstration of specific seroconversion by means of ELISA or serum-neutralization (SN) tests with indicator system, such as the neutralizing peroxidase-linked assay (NPLA). Confirmation of the diagnosis was made by the EU Reference Laboratory at Hanover, Germany with Real Time PCR method.

The purpose of this article is to describe the clinical, immunological, pathological, histopathological and molecular findings and that arose from an outbreak of CSF in Israel in a close-cycle pig holding facility in Northern Israel close to the Lebanese border.

MATERIALS AND METHODS

Samples

Two dead domestic pigs from the infected farm were examined at post-mortem. Tissue samples were collected from the spleen, tonsils, lymph nodes, kidneys, brain and intestine for molecular, virological and histopathological tests. For histopathology the various organs immersed in 10% neutral buffered formaldehyde (NBF) were embedded in paraffin, sectioned at 4 μm and stained with hematoxilin eosin (H&E). In addition specimens from clinically affected animals (among them domestic pigs, a dead pregnant sow, a wild boar) were sent to the Community Reference Laboratory (CRL), Hannover, Germany, for confirmation of CSFV. The details of samplings and of the results are presented in Table 1 below.

RT-PCR

Total RNA collected from tonsils, spleen, lymph nodes and kidneys of domestic pigs and a wild boar was extracted with TRI REAGENT T 118 (TRI REAGENT, Molecular Research Center, Inc. Cincinnati, OH,USA) according to the manufacturer's instructions. For reverse transcription, 1 μL of RNA with 1 μL (100 pmol) of forward primer (8)

Table 1: Results of samples positive for CSF.

Origin	**Antibody – ELISA**			**Antigen – ELISA**		**RT-PCR CSF**	
Farm	*total*	*positive CSF*	*positive BVD*	*total*	*positive*	*total*	*positive*
Serum	24	12	0	0	0		
Organs homogenate				6	5	6	4
					1 doubtful		
Fetuses homogenate				1	1		
Wild boars							
Serum	1	1	nt	nt	nt		
Organs homogenate				1	1	2	2
Totals	25	13	0	8	7 + 1 doubtful	8	6

nt = not tested

was heated to 95ºC for 1 min, chilled on ice, and added to 20 µL of a reverse transcription reaction mixture containing reaction buffer (25 mM Tris-HCl, pH 8.3 at 42ºC, 25 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.25 mM spermidine), 250 mM each of four deoxynucleotides, 25 U of RNasin (Promega Corp., Fitchburg, WI. USA) and 10 U of AMV reverse transcriptase (Promega Corp., Fitchburg, WI. USA). After incubation at 42ºC for 90 min, 1 µL of cDNA product was added to 25 µL of PCR reaction mixture, (300 mM Tris- HCl, 75 mM $(NH_4)2SO_4$, 7.5 mM $MgCl_2$, pH 8.5) containing 100 ng of each primer, using Amplitaq (PerkinElmer Inc., Waltham, MA, USA) according to the manufacturer's instructions. The following thermocycling program was used: 5 min at 95ºC, 40 cycles of 30 sec at 94ºC, 1 min at 55ºC, and 90 s at 72ºC followed by final 10 min extension step at 72ºC.

Sequencing and Genetic Analysis

The 300 and 271 bp fragments of 5'UTR and E2 PCR products, respectively were visualized on 1.5 % agarose gels, purified with the GenElute™ Agarose Spin Column (Sigma) and sequenced with the Automatic Sequencer 3700 DNA analyzer (Applied Biosystems) according to the manufacturer's instructions. Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast/Blast.cgi) search of 150 bp of 5'UTR sequences confirmed the identification of CSF virus. For the phylogenetic analysis the nucleotide sequences were aligned by means of CLUSTAL X program. A phylogenetic tree of 190 bp of the E2 fragment was constructed by the neighbor–joining method; the distances were calculated by using the maximum composite likelihood with the computer program MEGA, version 3.1 (9). The reliability of the phylogenetic groupings was evaluated by bootstrapping with 1,000 replicates.

ELISA

Twenty-four blood samples, from sows with and without clinical signs, were subjected to the indirect ELISA for the presence of CSF virus antibodies. The tests revealed the presence of CSF anti-protein E2 antibodies (1, 5). The same blood samples were also subjected to the ELISA for antibodies against Bovine Diarrhea Virus (BVD) using the IDEXX BVDV Ab Test Kit (IDEXX Laboratories Inc., Westbrook, Maine, USA). Six organ homogenates from sows, one organ homogenate from an aborted fetus, and one organ homogenate from a wild boar were subjected to direct ELISA test for the detection of viral antigens of CSFV using the PrioCheck CSF-Ag kit (Prionics Lelystadt B.V., a subsidiary of Prionics, Zurich, Switzerland).

RESULTS

The outbreak occurred on a farm in the north of Israel, 3.5 km from the Lebanese border, in a close-cycle holding of 425 sows, 6 boars and about 2,000 growing/fattening pigs of various ages. The area in which the farm is located is recognized as a transit route for wild boars. The holding consisted of various isolated buildings.

The first clinical signs appeared in the building occupied by pregnant animals and boars, which is located about 70 m from those used for farrowing, weaning and growing/fattening. The farm had no fence or barriers. A tentative diagnosis of Classical Swine fever was suggested by clinical and necropsy evidence, observed initially as hemorrhagic lesions of internal organs, including kidneys.

Clinical Aspects

The first clinical signs appeared on February 15, 2009 (Day 1) in one sow recently inseminated with imported semen. The sow showed weakness, high temperature (over 41˚C) and erythema. The sow was diagnosed with Erysipelas and treated with antibiotics (combination of penicillin-streptomycin) to which there was no response. The following day the same symptoms appeared in two other sows, one of which died, in the same pen. Within a few days the entire pen was affected. Four days later the first abortions occurred, and other sows showed anorexia, erythema, lameness, and difficulty in rising and walking, particularly with regard to the hind legs.

On Day 5 several sows remained in lateral recumbency, with abdominal breathing, coughing, and vomiting, including a few cases of hemorrhagic vomit. Two boars showed similar signs, including intense scrotal redness. Seven sows died from Day 2 through Day 9. During the first 10 days clinical signs remained limited to pregnant sows; farrowing, weaning and growing/fattening animals did not show evidence of any specific problems. Blood samples were collected from clinically affected sows, and organ samples from necropsies of sows (lung, lymph nodes, tonsils, spleen, liver, kidney, central nervous system, muscle) and from aborted fetuses.

Gross Necropsy Findings

On Days 8 and 9, the Veterinary Services Department performed the first necropsies. Dead sows showed multifocal hemorrhage and skin cyanosis that in some cases affected the entire body surface (Figure 1). At necropsy hemorrhages and petechiae were observed in the abdominal cavity, thorax (parietal pleura) (Figure 2), and epicardium (Figure 3), and on the diaphragm. Lungs presented with interstitial pneumonia with hemorrhagic foci (Figure 4); in some cases, intense and extended fibrinous pleuritis, probably caused by secondary bacterial infection, was observed. Spleen enlargement was observed in a number of cases. Lymph nodes were congested and enlarged and jagged hemorrhages (so-called "geographic map") were presented at the cut surface. Multi-focal hemorrhagic petechiae were found on the kidney surface (so called "turkey egg" kidney) (Fig. 5) with cortical hemorrhages on the cut surface. In the small intestine multi-focal necrotic lesions were observed.

Between Day 14 and 17, in parallel with the described outbreak, two apparently sick wild boars were shot, close to the farm. In the course of a thorough examination of the area, veterinarians of the Israel Nature and Parks Authority found another 10 dead wild boars, apparently from various dates of death within a 4 km radius around the herd. Blood and organ samples were collected for diagnosis.

Laboratory Investigations

Laboratory investigations were carried out to exclude African Swine Fever (ASF), Epidermitis-Nephritis syndrome caused by Porcine circovirus 2 (PCV2), acute Pasteurellosis,

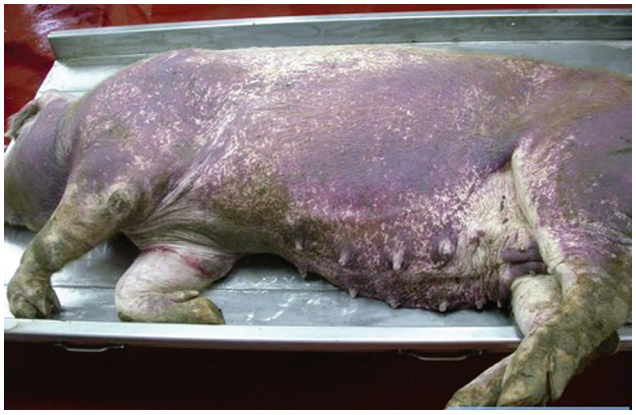

Figure 1. Pig carcass: Note the multifocal extensive hemorrhages and cyanosis.

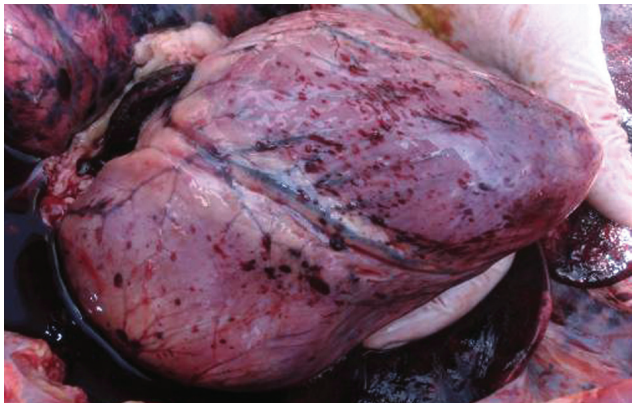

Figure 3. Heart: Multifocal petechial hemorrhages on the epicardium.

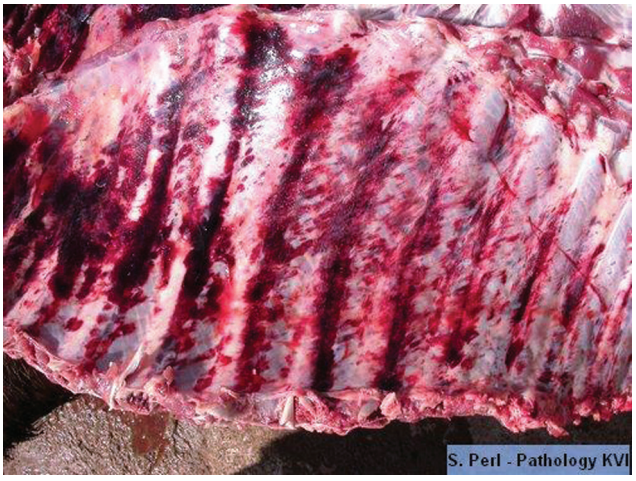

Figure 2. Multifocal petechial and echymotic hemorrhageson the parietal pleura.

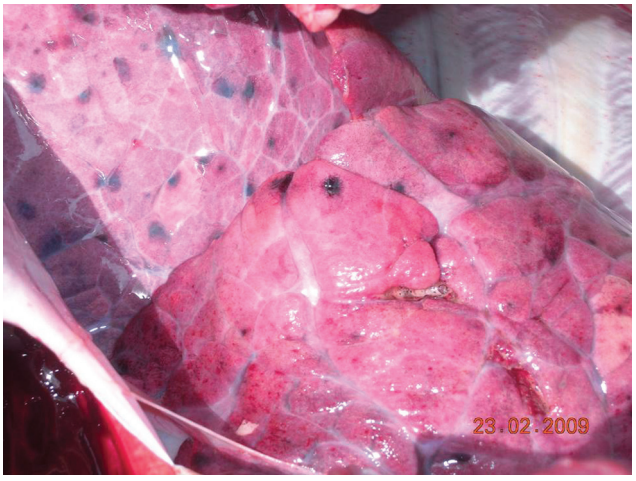

Figure 4. Multifocal petechial hemorrhages on the visceral pleura.

Figure 5. Kidney: Multifocal petechial hemorrhages on cut surface of the renal cortex.

Clostridium novyi infection (3), and intoxication by aflatoxins, mycotoxins, or ocratoxins (4). Porcine Respiratory and Reproductive Syndrome (PRRS) was not investigated, as Israel is free from PRRS.

Antigen – ELISA method

Out of eight samples tested, seven were found CSF-positive (Table 1). This test revealed CSF virus in whole blood, plasma, serum, and organs of wild boars.

Histological Examination

Histological examination of different organs and tissues revealed:

Tonsils: Necrotic focal tonsillitis, mononuclear infiltration and scarce neutrophilic cell infiltration.

Lymph nodes: lymphocyte depletion, necrotic foci in liver and pancreatic lymph nodes.

Spleen: Lymphocyte depletion, diffused sub-capsular and parenchymal hemorrhages.

Liver: Mononuclear cell focal infiltrations.

Kidney: Multi-focal hemorrhages and focal mononuclear cell infiltrations.

Intestine-Ileum: Necrotic foci on the mucosa, lymphocyte depletion, fibrin, accumulation of neutrophil cells and cellular debris.

Brain: Focal glyosis, perivasculitis, non-purulent meningo-encephalitis, necrotic foci.

Muscle: Perimysial hemorrhages, necrosis of muscle fibers (Fig. 6 a, b) (10).

Molecular Analysis

Samples were sent to the Kimron Veterinary Institute for diagnosis of CSFV and on day 13, CSF was confirmed by RT-PCR (Fig. 7). A 190 base-pair gene fragment of the E2 glycoprotein of the Israeli CSFV isolate was subjected to molecular analysis. CSFV presence was also confirmed by BLAST analysis of the 150 base pair (bp) sequence of the 5'-UTR which revealed a 98% homology between the Israeli sequences and CSFV sequences from GenBank (FJ290205, AY072924, AY568569, AF045071, AF045070, L42438, L42437 and AF045069).

Phylogenetic analysis of the Israeli sequences, GenBank sequences and the CRL database based on a 190 bp CSFV E2 glycoprotein fragment assigned the Israeli CSFV isolates from domestic pigs (DP1 and DP2) to subgroup 2.1. The

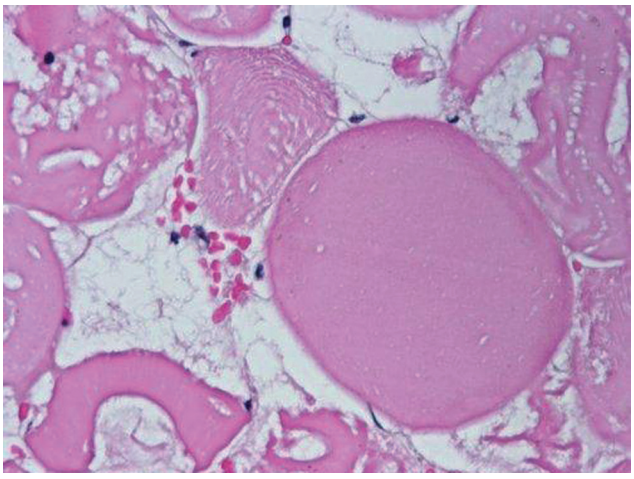

Figure 6a.

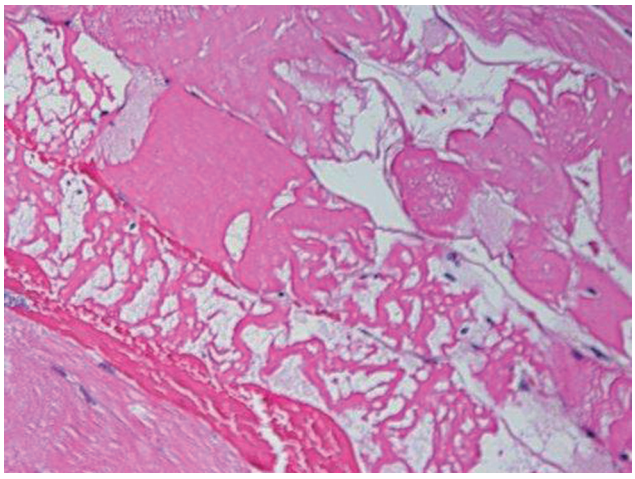

Figure 6b.

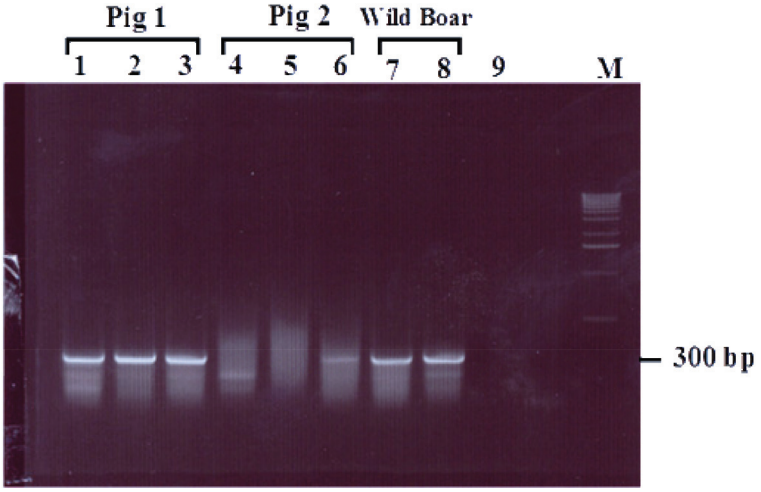

Figure 7. RT-PCR products of CSF virus 5'-UTR (300bp) using primers pest104F-pest402R for the amplification.

1-Spleen; 2-Lymph node; 3-Tonsil; 4-Spleen; 5-Lymph node; 6-Tonsil; 7-Spleen; 8-Tonsil; 9-Negative; M -Marker

DP1 and DP2 sequences of UTR and E2 have been deposited in the Genbank database (8).

Based on analysis of a 190 bp E2 fragment, the Israeli CSFV had 99% homology to CSFV sequences DQ907713, FJ529205, EF683606, FJ598642, FJ598610, which were isolated from pigs in China from 2005-2008. The deduced partial amino acid sequences of the E2 glycoprotein of subgroup 2.1 isolates from GenBank were compared with those of the Israeli CSFV isolate had 100% homology exclusively with Chinese isolates (8).

It seems possible therefore that the Israeli and Chinese CSFV strains originate from a similar source that probably was introduced into Israel by travelling between the two countries.

RT-PCR test, performed in Hannover confirmed the positive CSFV in the samples.

DISCUSSION AND CONCLUSIONS

Mortality attributed to CSF, from Day 1 to 36 totaled 103 sows out of 425 (24%), two boars out of six (33%), and an unknown number of abortions. Following serological and virology confirmation of CSF, the Veterinary Services decided to adopt a policy of culling all clinically sick animals and in parallel vaccinating all clinically healthy pigs. In spite of the fact that the farm where the isolate was made was isolated from other pig farms, cautiousness suggested to extend the vaccination to other herds located in the vicinity, a few kilometers away, and in proximity to the local pig slaughterhouse. For this purpose, a live attenuated vaccine was used: China strain "CL", at 100 PD_{50} / dose (2 ml) (11). Breeders were vaccinated twice with a 1-month interval; pigs for growing/fattening were vaccinated once, at age 7 days and upwards (11).

Although this vaccine has been demonstrated, in experimental vaccination-infection trials, to arrest the spread of a challenge virus in a short time (12), the present outbreak of CSF did not end promptly. Starting Day 45, clinical signs appeared in weaning and growing/fattening units. These clinical signs reappeared for about 1 month, and by Day 75, losses totaled 123 weaned piglets and 232 growers/fatteners, as well as a few additional sows. Total losses, through disease and culling, were 267 sows, 4 boars and 828 fattening pigs.

This CSF outbreak was the first reported incidence of CSF in Israel in the last 70 years (15), so the present report forms an important epidemiological contribution of CSF in the Middle East in general (16). The source of the outbreak is uncertain despite confirmation of CSF in wild boars.

The topography and design of the farm may have contributed to contacts between sows and wild boars. Some of these aspects include:

1. Groves and patches of vegetation around the farm could have provided a shelter for wild boars.
2. The layout of the farm, especially the pregnant sow unit was constructed as a large shed without fences and with wide entrances. Thus the possibility of direct access to the sows' troughs raises the possibility of entry of wild boars into the area.

The present CSF outbreak presented initially in the group of pregnant sows and then about 1 month later in the farrowing, weaning, growing and fattening units. It is difficult to evaluate whether the clinical signs in the different units represent a "late development" as described in the literature (8), resulting from a slow incubation of the virus in some animals (even those that were vaccinated) and then moving from the pregnant sow unit to farrowing units. The possibility of direct transmission by personnel through lapses of attention while executing direct prophylactic mea-

sures should also be considered or the transmission by aerosol droplets generated during high-pressure cleaning of the pregnant sow unit (11).

The use of vaccine prophylaxis was not able to completely prevent the transmission of the disease in units not primarily involved in the outbreak. However, as already mentioned, it is difficult to determine whether this was due to low efficacy or to "late development" of clinical signs in already infected animals. Furthermore it must be pointed out that the use of the vaccine does not enable discrimination between vaccinated and infected animals, as suggested by the OIE (12).

The positive findings of CSF in wild boars suggest the possibility of vaccine prophylaxis of wild boars by means of oral vaccines (1, 11). Clearly the upgrading of biosecurity measures using fencing and gates is of high priority for all farms in northern Israel in order to prevent the entrance of wild animals.

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