# *Serological evidence suggests that several Simbu serogroup viruses circulated in Israel*

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**Keywords**
Akabane disease,
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**Summary**
Viruses of the Simbu serogroup are arboviruses that are known to cause outbreaks of abortion, stillbirth and congenitally deformed neonates. This study presents the results of antibody screening of Simbu serogroup viruses in heifers born in Israel after October 2013, and in adult milking cows born before May 2012. Thirteen dairy cattle farms in five regions, and one sheep flock, entered this study. Serum samples that were found to be positive by ELISA were further tested by specific virus- neutralization test against a panel of Simbu serogroup viruses including Akabane, Aino, Sathuperi, Shamonda, and Peaton viruses. Antibody detection in lactating adult cows revealed that several viruses were circulating in Israel between 2008-2014. Moreover, during autumn 2014 the heifers became serum-positive after being exposed to more than one Simbu serogroup virus concurrently. The results of this study shed new light on Simbu virus infections in Israel, and may contribute to the epidemiology of the Simbu serogroup around the Mediterranean Basin in general.

## *Virus del sierogruppo Simbu in Israele: prove sierologiche ne suggeriscono la circolazione*

**Parole chiave**
Arbovirus,
ELISA,
Malattia di Akabane,
Virus del sierogruppo Simbu,
Test di neutralizzazione virale.

**Riassunto**
I virus del sierogruppo Simbu sono arbovirus che causano aborti, natimortalità o con malformazioni congenite. Questo studio presenta i risultati di uno screening anticorpale dei virus del sierogruppo Simbu in vacche da latte adulte nate prima di maggio 2012 e nelle giovenche nate dopo l'ottobre 2013; gli animali campionati in questo studio provenivano da tredici allevamenti di bovini da latte e da un gregge di pecore dislocati in cinque regioni in Israele. I campioni di sieri positivi all'ELISA sono stati ulteriormente testati con test specifici di neutralizzazione virale per i virus Akabane, Aino, Sathuperi, Shamonda e Peaton. Il rilevamento di anticorpi nelle vacche adulte in lattazione ha dimostrato che diversi virus del sierogruppo Simbu hanno circolato in Israele tra il 2008 e il 2014. Le giovenche, inoltre, che hanno sieroconvertito nell'autunno 2014, testimoniano un'esposizione a più di un virus del sierogruppo Simbu contemporaneamente. I risultati di questo studio fanno luce sulle infezioni da virus del sierogruppo Simbu in Israele e possono in generale contribuire ad una miglior comprensione dell'epidemiologia del sierogruppo Simbu intorno al bacino del Mediterraneo.

## Introduction

The Simbu serogroup is one of the largest serogroups within the *Ortobuyavirus* genus of the *Perbunyaviridae* family, comprising at least 24 viruses antigenically differing, but serologically related viruses (Kinney and Calisher 1981, Partsonson and McPhee 1985). Simbu serogroup viruses are arthropod-borne, and have been primarily isolated from, or detected in, *Culicoides* biting midges (Lee 1979, St. George *et al.* 1980, Cybinski 1984, Yanase *et al.* 2005, Balenghien *et al.* 2014, Kato *et al.* 2016a).

Akabane and Aino viruses (AKAV and AINOV) are the most studied among Simbu viruses, and are known to cause outbreaks of abortion, stillbirth and congenitally deformed neonates, when susceptible pregnant ruminants are infected (Markusfeld-Nir and Mayer 1971, Kurogi *et al.* 1975, Inaba *et al.* 1975, Uchinuno *et al.* 1988, Noda *et al.* 1998, Brenner *et al.* 2004a, Tsuda *et al.* 2004, Brenner *et al.* 2016). The recent emerging outbreaks of ruminant malformations in Europe were clearly associated with the Schmallenberg virus (SBV), a newly emerged Simbu serogroup virus first identified in Germany (Hoffmann *et al.* 2012, Goller *et al.* 2012, Wernike *et al.* 2014). Other Simbu serogroup viruses, including the Peaton, Shamonda, Sathuperi and Shuni viruses (PEAV, SHAV, SATV and SHUV), have also been implicated in the ruminant malformations (Partsonson and McPhee 1985, Yanase *et al.* 2012, Golender *et al.* 2015). The malformations seen at birth, known as congenital arthrogryposis-hydranencephaly (A-H) syndrome, are correlated with the pregnancy stage at which the dam first contracts the infection (Markusfeld-Nir and Mayer 1971, Kurogi *et al.* 1975, Inaba *et al.* 1975, Brenner *et al.* 2004a, Tsuda *et al.* 2004, Bayrou *et al.* 2014, Brenner *et al.* 2016). However, other clinical manifestations such as diarrhea, fever, reduced milk yield, and hypofertility (Kono *et al.* 2008, Hoffmann *et al.* 2012, Brenner *et al.* 2016) have also been reported in adult cattle. Thus, the diseases arising from Simbu serogroup virus infections have a significant adverse impact on animal health and welfare, the economy of livestock production, international trade, and movement of livestock worldwide (Dominguez *et al.* 2012, Hoffmann *et al.* 2012, Wernike *et al.* 2014, Martinelle *et al.* 2014).

Israel has been subjected to AKAV and AINOV infections in the past. Since 1969, there have been several outbreaks with clinical manifestations attributed to AKAV infections in ruminants (Markusfeld and Mayer 1971, Shimshony 1980, Brenner *et al.* 2004a, Brenner *et al.* 2016). In November 2014, SHUV (closely related to AINOV) was isolated from the brains of congenitally malformed lambs with A-H syndrome (Golender *et al.* 2015).

The aim of this study was to gain insight on the possible presence of Simbu serogroup antibodies in Israel. This work presents the results of antibody screening using specific virus neutralization test against a panel of five Simbu group species with sera collected at the end of summer 2014, in selected regions in Israel.

## Materials and methods

### Study design and sampling

In Israel, the summer and autumn are the main seasons of *Culicoides* spp. activity, depend on the climatic conditions and the geographic locations (Braverman and Chechik 1996). This corresponds with viral infection of the fetus (its dam) as clinical appearance of A-H syndrome in Israel usually occurs from December onward (in ovine) and around March-April (in bovine) (Shimshony 1980, Brenner *et al.* 2004a, Golender *et al.* 2015, Brenner *et al.* 2016). Moreover, the acute stage of bluetongue (BT) in sheep and epizootic hemorrhagic disease (EHD) in cattle begins in summer (Yadin *et al.* 2009, Brenner *et al.* 2010, Brenner *et al.* 2011, Kedmi *et al.* 2011, Bumbarov *et al.* 2012), also indicating high *Culicoides* activity during this season. Accordingly, serial serum sampling began in the first week of July until seroconversion was detected.

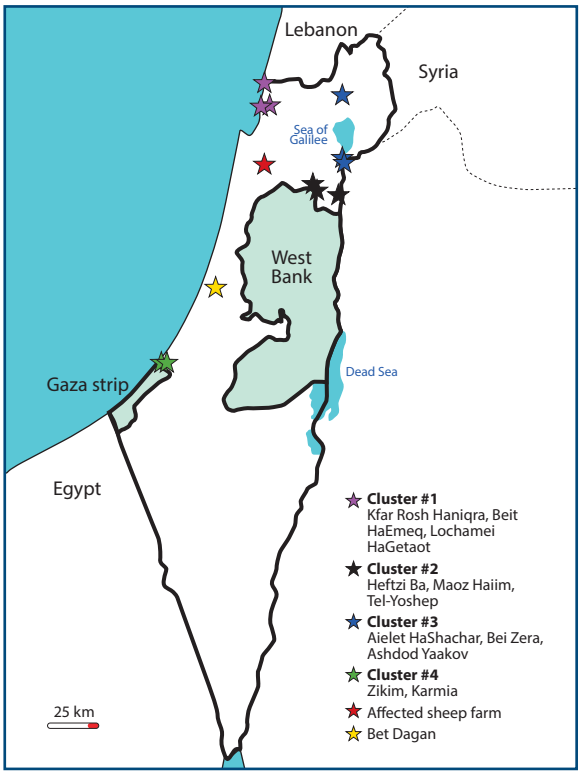


**Figure 1.** *Locations of the serosurvey study on Simbu serogroup viruses.*

For this study, 13 dairy cattle farms in 5 regions in Israel were selected. These farms are considered to be hotspots of *Culicoides* activity, where AKAV (Markusfeld and Mayer 1971, Brenner *et al.* 2004a, Brenner *et al.* 2016) and other *Culicoides*-transmitted viruses have been isolated (Brenner *et al.* 2010, Brenner *et al.* 2011, Yadin *et al.* 2008, Kedmi *et al.* 2011).

The selected regions are three valleys in northern Israel (Zvulun Valley - cluster #1; Jezreel Valley - cluster #2; Sea of Galilee - cluster #3), each with 3 collective dairy farms. Two farms were in the Southern Coastal Plain (cluster #4), and one experimental dairy farm in Bet Dagan, in the Central Coastal Plain where the Kimron Veterinary Institute (KVI) is situated (Figure 1). At each selected farm, 5 heifers and 10 milking cows were sampled (for a total of 75 and 140, respectively) at the beginning of the study. Thereafter, only the 5 selected heifers were retested serologically every 2 to 3 weeks until seroconversion occurred. By analyzing the adult cow stock, we expected to gain anamnestic information about previous cumulative exposures prior to the sampling. Conversely, the heifers, at an age of between 8 to 10 months, should have been serologically naïve in August/September 2014, and should serve as sentinels. Therefore, we chose heifers which were born after October 2013.

The main aim of the study was to find the etiology of the seroconversion in the selected heifers, if applicable. The Bet Dagan farm was tested because AKAV infection had been confirmed in it recently (2012) (Brenner *et al.* 2016). Thus, Simbu serogroup seroreactivity was assumed to have been positive in adult cattle before that date. Accordingly, only heifers under the age of 10 months were tested.

In addition, 5 serum samples from adult sheep were also included in this screening. The samples were collected from an infected sheep herd, where A-H syndrome had been observed, and SHUV had been isolated (Golender *et al.* 2015). Serum samples from known exposed dams were collected to examine possible epidemiological links between the anticipated heifers' seroconversion and the A-H syndromes reported in this sheep flock and in cattle and goat herds in late 2014 and early 2015 (Golender *et al.* 2015).

## Screening samples for Simbu serum-reactivity by ELISA

All samples collected during the study were tested by ELISA for the possible presence of Simbu serogroup antibodies. The IDEXX Schmallenberg Ab Test Kit (3097 Liebefeld-Bern, Switzerland), was used at the KVI in Israel. The ELISA procedure was carried out in accordance with the manufacturer's instructions. Since this ELISA kit is based on the nucleocapsid protein of Schmallenberg virus and detects antibodies against several Simbu serogroup viruses, selected serum samples that tested positive by ELISA were sent to Kyushu Research Station, National Institute of Animal Health, Japan for specific virus neutralisation test (VNT) analysis.

**Table I.** *Total distribution of neutralizing antibody titers to each Simbu serogroup virus for each sample that tested positive by ELISA. — cont'd*

| No. | Animal | Settlement (Cluster #) | Antibody titer | | | | |
|---|---|---|---|---|---|---|---|
| | | | Aino | Sathuperi | Akabane | Shamonda | Peaton |
| 1 | Adult cows | Kfar Rosh Haniqra (Cluster #1) | 8 | < 2 | 2 | 16 | < 2 |
| 2 | Adult cows | Kfar Rosh Haniqra (Cluster #1) | 4 | < 2 | < 2 | 16 | < 2 |
| 3 | Adult cows | Beit HaEmeq (Cluster #1) | 8 | < 2 | < 2 | 8 | < 2 |
| 4 | Adult cows | Lochamei HaGhetaot (Cluster #1) | 8 | < 2 | < 2 | 8 | < 2 |
| 5 | Adult cows | Lochamei HaGhetaot (Cluster #1) | 2 | < 2 | N/A | N/A | N/A |
| 6 | Adult cows | Heftziba (Cluster #2) | 16 | 32 | < 2 | 16 | 2 |
| 7 | Adult cows | Heftziba (Cluster #2) | 8 | < 2 | < 2 | < 2 | < 2 |
| 8 | Adult cows | Tel Joseph (Cluster #2) | 2 | 16 | < 2 | 4 | < 2 |
| 9 | Adult cows | Tel Joseph (Cluster #2) | < 2 | < 2 | < 2 | 8 | < 2 |
| 10 | Adult cows | Maoz Haim (Cluster #2) | 32 | 8 | 2 | 16 | < 2 |
| *11* | Adult cows | Beit Zera (Cluster #3) | 4 | 2 | 16 | 4 | < 2 |
| 12 | Adult cows | Beit Zera (Cluster #3) | 4 | < 2 | 32 | 8 | 4 |
| 13 | Adult cows | Ashdot Yaakov Meuchad (Cluster #3) | 2 | 4 | 128 | 16 | 32 |
| 14 | Adult cows | Ayelet Hashachar (Cluster #3) | 16 | 32 | 2 | 32 | < 2 |
| 15 | Adult cows | Ayelet Hashachar (Cluster #3) | 8 | 4 | 64 | 16 | 4 |
| 16 | Adult cows | Ziqim (Cluster #4) | > 256 | 128 | 64 | 32 | N/A |
| 17 | Adult cows | Ziqim (Cluster #4) | 64 | 16 | 32 | 8 | < 2 |
| 18 | Adult cows | Karmia (Cluster #4) | 16 | 2 | < 2 | 4 | < 2 |

continued

**Table I.** *Total distribution of neutralizing antibody titers to each Simbu serogroup virus for each sample that tested positive by ELISA. — cont'd*

| No. | Animal | Settlement ( Cluster #) | Antibody titer | | | | |
|---|---|---|---|---|---|---|---|
| | | | Aino | Sathuperi | Akabane | Shamonda | Peaton |
| 19 | Adult cows | Karmia (Cluster #4) | 64 | 16 | 16 | 4 | 32 |
| 20 | Heifer | Kfar Rosh Haniqra (Cluster #1) | 64 | 2 | 8 | < 2 | < 2 |
| 21 | Heifer | Kfar Rosh Haniqra (Cluster #1) | 32 | 8 | < 2 | < 2 | < 2 |
| 22 | Heifer | Kfar Rosh Haniqra (Cluster #1) | 16 | 4 | < 2 | < 2 | < 2 |
| 23 | Heifer | Lochamei HaGhetaot (Cluster #1) | 2 | 64 | < 2 | < 2 | < 2 |
| 24 | Heifer | Lochamei HaGhetaot (Cluster #1) | 32 | 4 | < 2 | < 2 | < 2 |
| 25 | Heifer | Lochamei HaGhetaot (Cluster #1) | 8 | 2 | 32 | 2 | < 2 |
| 26 | Heifer | Beit HaEmeq (Cluster #1) | 16 | 4 | < 2 | < 2 | < 2 |
| 27 | Heifer | Beit HaEmeq (Cluster #1) | 16 | 8 | < 2 | < 2 | < 2 |
| 28 | Heifer | Beit HaEmeq (Cluster #1) | 128 | < 2 | < 2 | < 2 | < 2 |
| 29 | Heifer | Beit HaEmeq (Cluster #1) | 64 | 4 | < 2 | < 2 | < 2 |
| 30 | Heifer | Tel Joseph (Cluster #2) | < 2 | < 2 | < 2 | < 2 | < 2 |
| 31 | Heifer | Tel Joseph (Cluster #2) | < 2 | < 2 | < 2 | < 2 | < 2 |
| 32 | Heifer | Heftziba (Cluster #2) | < 2 | < 2 | < 2 | < 2 | < 2 |
| 33 | Heifer | Heftziba (Cluster #2) | 32 | 2 | < 2 | < 2 | < 2 |
| 34 | Heifer | Maoz Haim (Cluster #2) | 16 | 2 | < 2 | < 2 | < 2 |
| 35 | Heifer | Maoz Haim (Cluster #2) | 64 | 2 | < 2 | < 2 | < 2 |
| 36 | Heifer | Maoz Haim (Cluster #2) | 32 | 4 | < 2 | < 2 | < 2 |
| 37 | Heifer | Maoz Haim (Cluster #2) | 64 | 2 | < 2 | < 2 | < 2 |
| 38 | Heifer | Beit Zera (Cluster #3) | 8 | 8 | 2 | 64 | < 2 |
| 39 | Heifer | Beit Zera (Cluster #3) | 64 | 16 | 2 | 32 | < 2 |
| 40 | Heifer | Beit Zera (Cluster #3) | 32 | 8 | N/A | N/A | N/A |
| 41 | Heifer | Ayelet Hashachar (Cluster #3) | < 2 | 32 | < 2 | < 2 | < 2 |
| 42 | Heifer | Ayelet Hashachar (Cluster #3) | < 2 | 64 | < 2 | < 2 | < 2 |
| 43 | Heifer | Ayelet Hashachar (Cluster #3) | < 2 | 32 | < 2 | < 2 | N/A |
| 44 | Heifer | Ayelet Hashachar (Cluster #3) | < 2 | 16 | < 2 | < 2 | < 2 |
| 45 | Heifer | Ashdot Yaakov Meuchad (Cluster #3) | 2 | < 2 | < 2 | 32 | < 2 |
| 46 | Heifer | Ashdot Yaakov Meuchad (Cluster #3) | 64 | 4 | < 2 | < 2 | N/A |
| 47 | Heifer | Ashdot Yaakov Meuchad (Cluster #3) | 16 | 2 | < 2 | < 2 | < 2 |
| 48 | Heifer | Ashdot Yaakov Meuchad (Cluster #3) | 16 | 2 | < 2 | < 2 | < 2 |
| 49 | Heifer | Ashdot Yaakov Meuchad (Cluster #3) | 2 | < 2 | < 2 | 32 | < 2 |
| 50 | Heifer | Ziqim (Cluster #4) | 32 | 4 | < 2 | < 2 | < 2 |
| 51 | Heifer | Ziqim (Cluster #4) | < 2 | < 2 | 2 | < 2 | < 2 |
| 52 | Heifer | Ziqim (Cluster #4) | 32 | 16 | 16 | 4 | < 2 |
| 53 | Heifer | Karmia (Cluster #4) | < 2 | 64 | < 2 | < 2 | < 2 |
| 54 | Heifer | Karmia (Cluster #4) | < 2 | 64 | < 2 | < 2 | < 2 |
| 55 | Heifer | Karmia (Cluster #4) | 32 | 4 | < 2 | < 2 | < 2 |
| 56 | Heifer | Karmia (Cluster #4) | < 2 | < 2 | < 2 | < 2 | < 2 |
| 57 | Heifer | Beit Dagan Exp. Farm | < 2 | < 2 | < 2 | < 2 | < 2 |
| 58 | Heifer | Beit Dagan Exp. Farm | < 2 | 16 | < 2 | < 2 | < 2 |
| 59 | Heifer | Beit Dagan Exp. Farm | 2 | 64 | < 2 | < 2 | < 2 |
| 60 | Sheep | Yoqneam | 16 | 2 | 16 | 32 | < 2 |
| 61 | Sheep | Yoqneam | 2 | < 2 | 2 | 16 | < 2 |
| 62 | Sheep | Yoqneam | 8 | 8 | < 2 | 4 | < 2 |
| 63 | Sheep | Yoqneam | 2 | 16 | 4 | 16 | N/A |
| 64 | Sheep | Yoqneam | 2 | 32 | 64 | < 2 | < 2 |

### Specific virus neutralization tests

A total of 64 serum samples, 59 cows (19 adults and 40 heifers) and 5 sheep were tested by specific VNTs (Table 1). The sera were subjected to VNTs against 5 viruses from the Simbu serogroup of the following respective strains: AKAV OBE-1, AINOV JaNAr28, SATV KSB-2/C/08, PEAV KSB-1/P/06, and SHAV KSB-6/C/02. After heat inactivation at 56°C for 30 min, the sera were serially diluted two-fold in serum-free Eagle's MEM, containing 10 µg/ml gentamicin sulfate from 1:2-1:256 in the 96-well microplates. Fifty microliters of serum dilution was mixed with an equal volume of virus inoculum containing 100 × the 50% tissue culture infective doses, and incubated at 37°C under 5% $CO_2$ for 1 hour. Then, 100 µl of the suspension of Hmlu-1 cells in GIT medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to each well, and incubated at 37°C under 5% $CO_2$ for 7 days. The antibody titer was calculated as the reciprocal of the highest serum dilution inhibiting the cytopathic effect. Samples were deemed positive if they had neutralizing antibodies to the viruses at a dilution of at least 1:8 (Kato *et al.* 2016b).

## Results

### Screening samples for Simbu serum-reactivity by ELISA

At time 0 (July 2014), all the milking cows tested positive by the ELISA. In contrast, all the heifers from all tested sites tested negative by the ELISA.

Subsequent ELISA tests confirmed that seroconversion occurred in the heifers within less than 6 weeks of their first testing.

### Simbu sero-group Virus neutralization tests

Antibodies against all of the five Simbu serogroup viruses tested (AINOV, AKAV, PEAV, SATV and SHAV) were detected in one or more of the serum samples from adult cows, heifers, and sheep. Their titer ranged from 1:8-1:256 (Table 1, Supplementary Table 1).

#### *Cluster #1*

The 5 selected ELISA-positive serum samples of adult animals (representing the anamnestic exposure to Simbu serogroup viruses in the Zvulun Valley/ Western Galilee) suggested that one or more ruminants in this region were exposed to AINOV and SHAV between 2008 and the sampling date (Table 1).

The 10 selected heifers' serum samples which should have specified the Simbu serogroup viruses responsible for serum reactivity found by ELISA indicated that anti-AINOV (8/10), anti-SATV (1/8) and anti-AKAV (1/8) antibodies were detected in ruminants in this region during the seroconversion process (Table 1).

#### *Cluster #2*

The 5 selected ELISA-positive serum samples of adult animals (representing the Jezreel Valley region) suggested that ruminants in this region were previously exposed to AINOV, SATV and SHAV (Table 1).

In the 8 selected heifers only anti-AINOV (5/8) antibodies were detected (Table 1). Three samples were negative by VNT despite being ELISA-reactive.

#### *Cluster #3*

The 5 selected ELISA-positive serum samples of adult animals (representing the regions around the Sea of Galilee) suggested that one or more ruminants in this region were previously exposed to all 5 tested viruses (e.i. AINOV, SATV, SHAV, AKAV, and PEAV) (Table 1).

In the 12 selected heifers anti-AINOV (5/12), anti-SATV(4/12) and anti-SHAV(3/12) antibodies were detected (Table 1).

#### *Cluster #4*

The 4 selected ELISA-positive serum samples of adult animals of cluster #4 representing the South Coastal Plain suggested that in this region ruminants were previously exposed to all 5 tested viruses (Table 1).

In the 7 selected heifers anti-AINOV (3/7) and anti-SATV (2/7) antibodies were detected. Two samples were negative by VNT despite being ELISA-reactive (Table 1).

#### *The experimental farm at Bet Dagan*

In the 3 selected heifers only anti-SATV (2/3) antibodies were detected (Table 1). One sample was negative by VNT despite being ELISA-reactive.

#### *Infected sheep herd*

Contrary to our expectations, in the 5 ELISA-positive serum samples of the clinically affected sheep herd from which SHUV had been isolated (Golender *et al.* 2015), four of the five tested adult sheep reacted to at least 2 different Simbu serogroup viruses (Table 1, Supplementary Table 1). In one sheep only anti-SHAV antibodies were detected.

## Discussion

The results of this study indicate that all tested ruminants had antibodies against one or more of the five Simbu serogroup viruses tested by ELISA and VNTs. Our results also show that the seroconversion detected in the naïve heifers by ELISA during summer/autumn 2014 was the result of more than one Simbu serogroup virus circulating simultaneously in Israel.

Prior to 2012, only AKAV had been linked to A-H syndrome in Israel (Shimshony 1980, Brenner *et al.* 2016). Our results reveal no evidence of AKAV in adult cows (2-8 years old) in two regions that have hitherto been considered AKAV 'hotspots'. These results are in accordance with a previous study conducted in Israel (Kalmar *et al.* 1975). Moreover, there is little evidence of current infection with AKAV, as antibodies against AKAV rarely appeared in the seroconversion process of the selected heifers during summer/autumn 2014 (Table 1). The unexpected absence of AKAV exposure at Bet Dagan since 2013 (Table 1) also lends further support to this 'anomalous' finding. Therefore, we postulate that AKAV is not the main Simbu serogroup virus present in Israel, as previously thought, but is only one of several etiological agents from this serogroup circulating in Israel.

Even though VNTs are considered to be the 'gold standard' for the assessment of other viruses, it is recognized that cross-reactivity between viruses belonging to the Simbu serogroup should be expected and taken under consideration during the interpretation of such serological results. The differences between these viruses can be recognized to at least seven different species by specific serological tests such as cross-neutralization test and cross-heamagglutination-inhibition tests (Plyusnin *et al.* 2012). In addition, the close relationships between some of these viruses can also be revealed in their current taxonomic classification where the species Sathuperi virus (SATV) includes Sathuperi, Douglas and Schmallenberg viruses; species Shamonda (SHAV) virus includes Shamonda, Peaton (PEA) and Sango viruses; species Akabane virus (AKAV) includes Akabane, Tinaroo and Sabo viruses; species Shuni virus (SUHV) includes Aino and Shuni viruses and species Simbu virus includes only Simbu virus (Goller *et al.* 2012).

Recognizing these limitations, five viruses representing different Simbu species were chosen for this study. Accordingly, several postulations are emerging from our data.

The antibodies detected in the adult lactating cows indicate that besides AKAV, AINOV and SHUV, which have already been documented (Brenner *et al.* 2004a, Golender *et al.* 2015, Brenner *et al.* 2016), other viruses of the Simbu serogroup (i.e. SATV and SHAV) were probably also circulating in Israel between 2008 and 2014.

Notably, antibodies to PEAV were detected in two adult cows. Since cross reactivity between PEAV and Sango virus (SANV) has been reported before (Kinney and Calisher 1981), our results might indicate for the first time, a remote circulation of either of these viruses in Israel. While preparing this manuscript, PEAV genomic material was detected in a calf exhibiting micro-hydranencephaly, supporting the assumption that PEAV is currently (2017) circulating in Israel (data not shown). Similarly, the VNT results from the naïve heifers in the summer/autumn of 2014 also revealed concurrent seroconversion of mixed Simbu serogroup viruses (mainly to AINOV and SATV, but also to SHAV and AKAV) in Israel. SHAV has been shown to have cross reactivity with AKAV (Kinney and Calisher 1981). Nonetheless, it is likely that each of these viruses was circulating in Israel as there were both adult cows and heifers with high antibody titers to a single virus and very low or no antibodies to the other viruses (Table 1). In addition, samples from known affected sheep of the flocks from which SHUV had been isolated (Golender *et al.* 2015) yielded VNT antibodies to SAT, SHA and AKA viruses. Consequently, this infected sheep herd provides indications that the Israeli' ruminants in 2014 were indeed exposed to more than one Simbu serogroup viruses, concurrently or sequentially.

The findings of this study are of epidemiological significance, not only because they shed new light on Simbu serogroup virus infections in Israel, but also because mixed viral infections may create the baseline conditions for viral re-assortment, as previously demonstrated by Yanase and colleagues (Yanase *et al.* 2010, Yanase *et al.* 2012). The co-existence of several different Simbu viruses in specific 'pockets' in Israel may indicate that Israel (and by extension the Middle East) may have functioned as a hotspot for virus evolution and/or re-assortment. Similar re-assortment has been observed in the BTV mixed infections. Probably such processes were responsible to generate novel viruses leading to various clinical syndromes observed at infected sites in different geographical regions in Israel (Brenner *et al.* 2010, Brenner *et al.* 2011). Nevertheless, to further improve our understanding of the epidemiology of the Simbu serogroup and arboviruses in general in Israel, this work should be complemented by entomological studies, in parallel with sero-specific (namely, VNT) surveillance and genomic detection (PCR or virus isolation) in insect vectors and/or mammalian hosts.

Finally, from closer examination of the VNT results, it is fairly apparent that each individual cluster/location presented a distinct site-specific serum reactivity

profile (Table 1). Even locations that had been thought to belong to the same cluster, including those within a radius of only 5 to 10 kilometers, differed from each other. The mechanism behind the cyclical arbo-disease pattern, where a given Simbu virus disappears from a certain location for relatively long periods, only to reappear at the same infected site several years later (Brenner *et al.* 2004b), remains a mystery. It should be noted that Mathew and colleagues (Mathew *et al.* 2015) published a similar study in Tanzania, with similar methodology and conclusions. However, unlike Tanzania, Israel is small and crowded, with extensive agricultural areas and animal farms that create a very high density of ruminant population. Therefore, the different distributions of Simbu serogroup observed in this study, even within a radius of 5-10 km, may also indicate that 'ecological pockets' such as the ones chosen for our study indeed exist. Investigating these 'ecological pockets' may improve our understanding of the arbo-spreading transboundary diseases.

## Conclusions

This study shows for the first time, that Israeli' ruminants are exposed to a mixed infection of more than one Simbu serogroup viruses, circulating in Israel concurrently or sequentially. This is also the first serological indication of Simbu serogroup viruses from the species Shamonda and Sathuperi in Israel. Due to potential cross reactivity, it is possible that viruses from the Simbu serogroup, other than the ones included in the test, are also present. Isolation and further genetic characterization of Simbu serogroup viruses, including isolates from vectors and ruminants from different geographical regions, will be essential for understanding the molecular epidemiology and evolution of these viruses.

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## *Annex 1*

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**Supplementary Table I.** *Distribution of neutralizing antibody titers to each Simbu serogroup virus.*

| Animal | Virus | Antibody titers | | | | | | | Positive sera/ tested sera (%) |
|---|---|---|---|---|---|---|---|---|---|
| Adult cow | AINOV | 7 | 5 | 3 | 1 | 2 | 0 | 1 | 12/19 (63.2) |
| | SATV | 12 | 1 | 3 | 2 | 0 | 1 | 0 | 7/19 (36.8) |
| | AKAV | 11 | 0 | 2 | 2 | 2 | 1 | 0 | 7/18 (38.9) |
| | SHAV | 5 | 5 | 6 | 2 | 0 | 0 | 0 | 13/18 (72.2) |
| | PEAV | 15 | 0 | 0 | 2 | 0 | 0 | 0 | 2/17 (11.8) |
| Heifer | AINOV | 17 | 2 | 6 | 8 | 6 | 1 | 0 | 23/40 (57.5) |
| | SATV | 25 | 4 | 4 | 2 | 5 | 0 | 0 | 15/40 (37.5) |
| | AKAV | 36 | 1 | 1 | 1 | 0 | 0 | 0 | 3/39 (7.7) |
| | SHAV | 35 | 0 | 0 | 3 | 1 | 0 | 0 | 4/39 (10.3) |
| | PEAV | 37 | 0 | 0 | 0 | 0 | 0 | 0 | 0/37 (0.0) |
| Sheep | AINOV | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 2/5 (40.0) |
| | SATV | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 3/5 (60.0) |
| | AKAV | 3 | 0 | 1 | 0 | 1 | 0 | 0 | 2/5 (40.0) |
| | SHAV | 2 | 0 | 2 | 1 | 0 | 0 | 0 | 3/5 (60.0) |
| | PEAV | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0/4 (0.0) |

AINOV = Aino viruses; SATV = Sathuperi viruses; AKAV = Akabane viruses; SHAV = Shamonda viruses; PEAV = Peaton viruses.