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The seroepidemiology of pestivirus infection in sheep in Afyonkarahisar province of Turkey and the analysis of associated risk factors

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ABSTRACT: Border disease virus (BDV) is a pestivirus that causes considerable economic losses in the sheep industry due to its effect on breeding and health. This study's goal was to determine the seroprevalence of pestivirus infection, the ratio of persistently infected (PI) animals, and risk factors associated with the disease in sheep flocks between 2019-2020. To this end, 460 blood serum samples collected from eight sheep flocks were examined using commercial Enzyme-Linked Immuno-Sorbent Assay (ELISA) test kits to determine the presence of antibodies (Ab) and antigens (Ag) against pestiviruses (bovine viral diarrhoea virus, border disease virus). Risk factors associated with pestivirus infection were statistically evaluated in terms of significance in the logistic regression model according to epidemiological data and information obtained from flock owners. Individual seropositivity was analyzed by Generalized Estimating Equations (GEEs) for associated responses. The overall apparent animal level seroprevalence was estimated to be 24.57% (95% CI: 20.85-28.7). The overall true seroprevalence was calculated to be 25.51% (95% CI: 21.65-29.60). The rate of positive sheep in each flock varied between 8.33-57.14%. The ratio of PI sheep among the 460 animals tested was found to be 0.43%. The relationship between the age groups was statistically significant (p -value: $0.0002 < 0.05$; χ^2 : 13.15). Management type, age, the presence of cattle in the farm, landscape and the status of other clinical diseases were identified as important risk factors associated with individual pestivirus seropositivity. The results of this study indicate that it will contribute to the creation of national control eradication and monitoring plans and the development of strategies and that the potential risk of sheep as a pestivirus reservoir, especially for cattle that use common pastures, should be considered in future studies.

Keywords: pestivirus, sheep, BDV, risk factors, epidemiology, Turkey

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INTRODUCTION

Pestiviruses infect small and large ruminant species, causing significant economic losses worldwide due to their effect on breeding and health (Krametter-Froetscher et al., 2007). Bovine viral diarrhoea virus 1 (BVDV1), bovine viral diarrhoea virus 2 (BVDV2), classical swine fever virus (CSFV), and border disease virus (BDV) are in the Pestivirus genus in the Flaviviridae family. Since 2017, the International Committee on Virus Taxonomy (ICTV) has renamed the aforementioned four species as Pestivirus A, B, C and D, respectively, and in addition to these, Pestivirus E (pronghorn pestivirus), Pestivirus F (Bungowannah virus), Pestivirus G (giraffe pestivirus), Pestivirus H (HoBi-like pestivirus), Pestivirus I (Aydin-like pestivirus), Pestivirus J (rat pestivirus), and Pestivirus K (atypical porcine pestivirus) were added, and a total of 11 species were identified (Simmonds et al., 2017). BDV is in genetic and antigenic affinity with CSFV and BVDV (Marco et al. 2007; Feknous et al., 2018).

BDV infection is commonly observed in sheep, and it has been reported that it can also cause disease in cattle, goat and pig species (Paton et al., 1995; Braun et al., 2014; Schweizer and Peterhans, 2014; Feknous et al., 2018). BDV is the primary cause of congenital infections in sheep, and it can cause acute, foetal, and persistent infections. The main route of transmission of pestiviruses is horizontal transmission through transiently infected and PI animals. Furthermore, BVDV-1, BVDV-2, BDV, CSFV, and HoBi-like pestiviruses can be transmitted vertically. The disease usually causes abortions in pregnant animals, low birth weight and dog-hair appearance in lambs (Van Campen and Frolich 2001; Monies et al., 2004; Kittelberger and Pigott, 2008; Şevik 2018).

Prevalence studies constitute a prerequisite for control and eradication programs, and pestiviruses are among the main causes of reproductive problems and immune system effects in cattle and sheep in most countries (Radostits et al., 2007). Understanding the relationship between seroprevalence and PI animals can be used to assess the immunization potential of sheep not exposed to disease prior to breeding (Nettleton, 2000; Berriatua et al., 2004). An important aspect of pestivirus control and eradication programs implemented in various continents and countries of the world is the detection and eliminating of PI animals (Lindberg and Alenius, 1999; Berriatua et al., 2006). PI animals are infected during early embryon-

ic and fetal development, and unlike individuals who become infected in the late period of pregnancy, they are seronegative and shed the virus throughout their lives. As a result, detecting PI animals is epidemiologically important since PI animals are the most significant source of infection for other animals (Houe, 1999). ELISA kits are preferred in pestivirus studies due to their advantages such as fast screening of many samples, sensitivity and being economic (Gonzalez et al., 2014; Hanon et al., 2018). The presence of viral antigens in PI animals can be detected by the ELISA method, which is a fast technique that is most commonly used in blood samples (Sandvik, 2005; Avci and Yavru, 2014).

In Turkey, sheep breeding is carried out in the form of large, medium, small flocks or a traditional family business within technical and economic opportunities according to the climate and natural conditions. There is no pestivirus vaccination program for small ruminants in Turkey. Although there are studies on the seroepidemiology of pestiviruses in sheep flocks in Turkey, there are almost no studies on the determination and analysis of risk factors associated with the disease. In the present study, it was aimed to predict the seroepidemiology and spread of pestivirus in sheep flocks in Afyonkarahisar province of Turkey and determine associated risk factors. It is expected that such epidemiological studies will contribute to the reduction of the prevalence of pestivirus infection, the creation of control eradication and monitoring plans for the country, and the development of strategies.

MATERIAL AND METHODS

Study area and description

Afyonkarahisar province is located between 37° 45 and 39°17 north latitude and 29° 40 and 31°43 east longitude. In the Master Plan of the Ministry of Agriculture and Forestry, the province is divided into four agro-ecological sub-regions, considering the agricultural diversity and climate data (I. Sub-Region, II. Sub-Region, III. Sub-Region, IV. Sub-Region) (Fig. 1). Although Afyonkarahisar province is located in the Aegean Region, it is under the influence of the continental climate, and winters are heavily snowy, and summers are hot and dry. The region of the province ranks first in the Aegean region with the number of small ruminants of 1 045 000 and has an important position in terms of animal husbandry potential (TUIK, 2020).

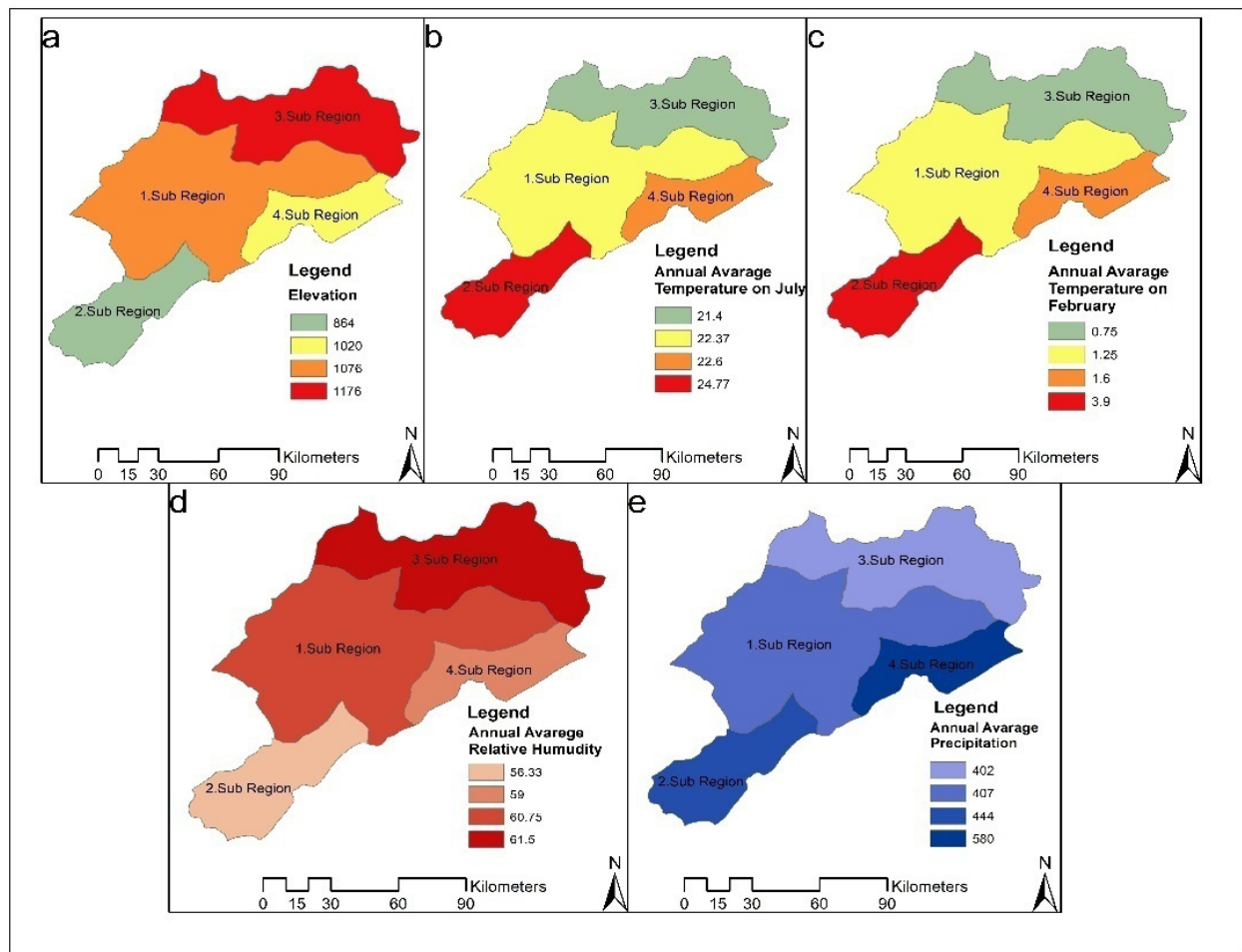


Figure 1. The study area

Samples, data management and analysis

In this study, the random sampling method was chosen for 460 animals from eight sheep flocks that were not vaccinated against pestivirus in the region of Afyonkarahisar province between 2019-2020. The required minimum within-flock sample size was computed on the basis of the epidemiologic research (Thrusfield, 2005; Dohoo et al., 2010). Four hundred sixty blood samples were drawn from the *vena jugularis* of the sheep selected, 10 ml were taken into blood tubes and centrifuged at 3000 rpm for 20 min. The serum obtained after this procedure was transferred to 1.5 ml sterile Eppendorf tubes. It was stored at -20°C until being tested. Approval for the study protocol was obtained from Pamukkale University Animal Experiments Ethics Committee (Report No: PAUHADYEK-2019/36). Sheep owners were interviewed directly during the same visit with drawing the blood sample to obtain information on epidemiological data. Flock size, composition, animal age, animal movements, the presence of goats and cows

in the enterprise, farming management (nomadic-sedentary), health status (abortion, stillbirth, joint disorders, nervous symptoms, other diseases, etc.) data were saved in Microsoft Excel spreadsheets.

Antibody ELISA

The serum samples collected were examined by indirect ELISA (IDEXX BVDV/MD/BDV p80 protein Ab IDEXX Laboratories, Westbrook, Maine, USA) in terms of the presence of antibodies against pestivirus. The antibody ELISA utilized to test serum samples is an ELISA based on the highly conserved p80 (non-structural pestivirus polypeptide) protein. The antibody ELISA test was carried out following the procedure reported by the manufacturer. The Optical Density (OD) values of the test results were read in an ELISA reader at 450 nm wavelength. The percentage inhibition (PI) values of the test sera were calculated according to the formula presented below by comparing them with the negative control.

$S/N\% = \frac{OD_{S450}}{OD_{N450controlmean}} \times 100$
 where OD_{450} is the optical density at 450 nm of the sample (S) and negative control mean (N). The samples with the values of 0.50 and above were evaluated as negative, between 0.40 and 0.50 as suspicious, and below 0.40 as positive.

Antigen ELISA

The commercial BVDV antigen ELISA (IDEXX BVDV Ag/Serum Plus IDEXX Laboratories, Westbrook, Maine, USA) kit was utilized to detect the presence of pestivirus antigens. The test, based on the principle of detecting viral antigens in study samples, was performed according to the procedure reported by the manufacturer. Ag ELISA results were calculated for each sample.

$$S-N = OD_{S450} - OD_{N450controlmean}$$

where OD_{450} refers to the optical density at 450 nm of the sample (S) and negative control mean (N). The samples with the values of 0.30 and below were evaluated as negative and above 0.30 as positive. ELISA-antigen positive animals were sampled again two weeks later to verify whether these animals corresponded to PI status or suffered a transient infection.

Statistical analysis

Data processing was carried out in the R program (R Core Team, 2018). Descriptive statistics were performed to determine the ratio of flocks with respect to the corresponding ratios of seropositive animals. GEE was performed by utilizing the R “geepack” package (R Package geepack for Generalized Estimating Equations) to estimate the overall within-flock seroprevalence. Potential risk factor parameters associated with pestivirus infection were compared in

terms of seroprevalence. Logistic regression analysis was conducted to determine the impact shares of risk factors thought to be effective on pestivirus infection. The disease was defined as positive (1) or negative (0) according to the serological test results of 460 samples included in the application in the study. According to the serological test results, it was defined as a categorical dependent variable (Y) that can take a value with two results. The interpretation of the model was carried out using odds ratios (OR). Each of the risk factors was examined with seropositivity using contingency tables. Fisher’s exact test and chi-square test were used to examine the relationships between response variables and explanatory variables. The value of $p < 0.05$ was accepted as statistically significant. Factors associated to BDV seropositivity with a $p < 0.10$ were included in the multiple model. Multiple logistic regression was used to assess the associations of potential risk factors with BDV seropositivity. The final model fit was assessed with Hosmer-Lemeshow goodness-of-fit test (Dohoo et al., 2010)

RESULTS

Of the 460 sera tested by antibody ELISA, 113 samples were detected as seropositive. The apparent overall within-flock seroprevalence was estimated to be 24.57% (95% CI: 20.85-28.7) based on the GEE model. The rate of positive sheep in each flock varied between 8.33-57.14%. The overall true seroprevalence was calculated to be 25.51% (95% CI: 21.65-29.60), considering the sensitivity (96.3%) and specificity (100%) of the Ab-ELISA test kit. The relationship between the animals’ age groups (1-3 years old; >3 years old) was found to be statistically significant (p -value:0.0003<0.05; χ^2 :13.15). Detailed results are presented in Table 1.

Table 1. Prevalence of antibodies to BDV with 95% CI according to GEE model

Flock	No of sampled animals	positive	prevalance%	95% CI
I.	48	4	8.33	3.29-19.55
II.	56	32	57.14	44.14-69.23
III.	60	12	20.00	11.83-31.78
IV.	58	10	17.24	9.64-28.91
V.	54	17	31.48	20.68-44.74
VI.	58	8	13.79	7.16-24.93
VII.	64	14	21.88	13.5-33.43
VIII.	62	16	25.81	16.55-37.88
	460	113	24.57	20.85-28.7

Of the 460 serum samples examined by antigen ELISA, 9 (1.96%) were determined to be positive for the presence of antigen, they were negative for the presence of BDV-specific antibodies. The said animals were resampled 14 days later. Of the 9 animals which were positive for the BDV antigen in the first sampling, 2 were positive in the second sampling, and 2 samples were negative for BDV-specific antibodies. It was determined that these PI animals had moderate to high seropositivity, and there were more animals aged > 3 years. Two sheep (0.43%) determined to be PI were removed from the enterprises they belonged to.

In the study, the differences in the ratio of seropositive animals were analyzed in terms of the animal purchase, abortion histories, sub-region conditions, flock management, flock composition, the presence of other clinical diseases in the flock (pneumonia, gastrointestinal, respiratory problems, etc.) variables.

The detected differences between the variants in question were statistically assessed following epidemiologic principles. The odds ratio was calculated to be 1.56 times higher in terms of flock management (the nomadic status compared to the sedentary status), the odds ratio was calculated to be 0.45 times higher in terms of the age range (animals aged above 3 years compared to animals in the 1-3 age range), the odds ratio was calculated to be 1.57 times higher in terms of the presence of cows in the flock (yes/no), the odds ratio was calculated to be 1.54 times higher in terms of the presence of other clinical diseases in the flock (yes/no). Detailed results involving odds ratios for these risk factors and the analysis of risk factors associated with seropositive BD are presented in Table 2. The multiple regression logistic model determined that age, flock effect, the presence of cattle in the farm, and the status of other clinical diseases are risk factors for BDV infection (Table 3).

Table 2. Risk factors related to seropositive for Border Disease

Factors	Categories	positive	negative	p-value	OR	95% CI
Agro-ecological sub region	I.	24	75	0.01	0.50	0.28-0.88
	II.	55	86	0.06	2.09	0.95-4.58
	III.	11	72	0.15	1.58	0.83-3.01
	IV.	23	114			
Other clinical diseases	yes	58	141	0.04	1.54	1.00-2.36
	no	55	206			
Age	1-3 years	46	209	0.0003	0.45	0.29- 0.69
	>3 years	67	138			
Flock status	sheep with cattle	57	136	0.03	1.57	1.03-2.42
	no cattle	56	211			
	sheep with goat	36	138	0.13*	0.70	0.45-1.11
	no goat	77	209			
	mixed (goat or cattle)	61	143	0.01	1.67	1.09-2.56
	sheep only	52	204			

Table 3. Factors included in the multiple logistic regression analysis

Variable	Categories	b	SE	OR	95% CI	p-value
Flock effect	nomadic*					
	sedentary	-0.53	0.23	0.58	0.37-0.92	0.02
Age	1-3 years*					
	>3 years	0.83	0.26	0.61	1.46-3.54	< 0.001
Other clinical diseases	Yes*					
	No	0.46	0.23	1.58	1.02-2.45	0.03
Flock status	sheep with cattle*					
	no cattle	-0.560	0.24	0.57	0.36-0.89	0.01

*Reference category

b: regression coefficient

SE: Standard Error

OR:Odss Ratio

DISCUSSION

Seroprevalence studies are essential in determining the exposure to pestiviruses in a flock, detecting active infection in a flock, and in the preparation of control eradication programs and the creation of implementation strategies (Houe, 1999). Different methods are used for the diagnosis of pestivirus infection, and blocking and indirect ELISA methods are commonly used serological tests (Sandvik, 2005). The detection of antibodies by ELISA in ruminants after pestivirus infection provides reliable results in terms of seroconversion (Feknous et al., 2018). ELISA-based tests have been used in studies to determine the seroepidemiology of pestivirus infection in small ruminants in various countries and continents of the world (Krametter-Froetscher et al., 2007; Mishra et al., 2009; Avcı and Yavru, 2014; Feknous et al., 2018).

Seroprevalence rates in sheep vary between 0-50% on a country basis (Nettleton et al., 1998). In this study, the apparent overall within-flock prevalence was determined to be 24.57% (95% CI 20.85-28.7). The overall true seroprevalence was calculated to be 25.51% (95% CI 21.65-29.60). The study results are similar to the individual animal seroprevalence findings in the studies conducted in Austria (29.4%), India (23.4%), Spain (17.9%), Iran (21.20%), and the inland and coastal zones of Turkey (18.94%) (Mainar-Jaime et al., 1999; Okur-Gumuşova et al., 2006; Mishra et al., 2009; Krametter-Froetscher et al., 2010; Muhammadi et al., 2011). However, a various seroprevalence rate of 51-90.9% was observed at the individual level in some studies in other countries (Berriatua et al. 2004; Gaffuri et al., 2006; Schiefer et al., 2006; Valdazo-Gonzalez et al., 2006). These variable seroprevalence rates can be attributed to many factors such as the management system specific to the country in general, the biosecurity measures at the entry of new animals into farms, poor housing conditions, insufficient information about the disease, and the lack of screening tests at regular intervals.

The prevalence of PI sheep or viremic sheep worldwide is between 0.3% and 20%. The prevalence of PI is 0.32% in Austria and varies between 0.24-0.6% in Spain (Valdazo-González et al., 2008; Martin et al., 2015). The PI sheep rate in this study was determined to be 0.43%. The reason why this rate is lower compared to the rates in some other studies can be explained by the fact that there are animals slaughtered for economic purposes and this is a factor that reduces the probability of detecting PI animals

during sampling (Valdazo-González et al., 2006). Another reason may be due to the time the infection has occurred in the flock. Furthermore, the circulation of the infection in the field is provided by acutely and persistently infected animals. Although the number of PI animals is usually low, it ensures the continuity of the virus in the flock. Therefore, it is very important to check all animals in the flock periodically from virological aspects and determine whether newborns are persistently infected.

According to the correlation test between seropositivity and seronegativity in the distribution of the age groups in the study, it was detected that positivity increased with increasing age. A higher seroprevalence rate was detected in sheep aged > 3 years old (32.68%) compared to sheep aged between 1-3 years (18%), which is explained by their long-term exposure to pestiviruses. The high seroprevalence rates observed in the study in animals aged > 3 years can be explained by the increased chance of exposure to the virus in older animals compared to younger animals (Berriatua et al., 2004; Mishra et al., 2008). The fact that the sheep sampled in the study are older than one year, the decrease in maternal antibodies until this age, and the presence of the antibodies detected since sheep in Turkey are not vaccinated against pestiviruses depend on the direct exposure to pestiviruses.

BDV prevalence and its association with various risk factors were analyzed. An investigation of risk factors associated with the disease has become significant in taking control measures for infection. In the current research, the rates of seroprevalence varying between the sub-regions were also observed. Seroprevalence (P) was lower in sub-region III ($P=13.25\%$; OR:2.09; 95% CI: 0.95-4.58) compared to sub-region II (39%). The low seroprevalence (13.25%) observed in sub-region III can be explained by the fact that most of the flocks in that region are immobile. It can also be associated with the presence of mountainous areas in this sub-region. Such conditions may be related to the absence of contact with infected flocks and the fact that transmission also depends on the degree of contact between animals (Nettleton et al., 1992; Feknous et al., 2018).

A lower prevalence was detected in sheep flocks in the sedentary system ($P=20.18\%$; OR: 1.56; 95% CI: 1.01-2.41) compared to transhumant flocks ($P=28.14\%$). The high prevalence observed in transhumant flocks can be explained by the fact that flocks are on the move to reach more grazing land and there

may be contact with other flocks. It has also been reported in previous studies that transhumance has a significant effect as a risk factor at the individual level (Tabbaa et al., 1995; Krametter-Froetscher et al., 2007; Martin et al., 2015).

In some of the pestivirus seropositive sheep flocks, a history of clinical symptoms including pneumonia, weakness, enteritis, a history of reproductive failure, abortion, stillbirth and birth of small and weak lambs is based on breeders' statements. According to the statements of flock owners, it has been observed that seroprevalence is higher in flocks with other clinical diseases (diarrhea, respiratory problems, weak lambs) in the flock history in comparison with flocks without clinical symptoms and the odds ratio increases in direct proportion ($P=29.14\%$; OR:1.54; 95% CI: 1.00-2.36). Seropositivity was determined to be statistically significant (p -value :0.04<0.05; χ^2 :3.97). In this context, pestivirus infections in sheep should not be ignored because they may cause the suppression of the immune system against common pathogens that cause economic losses in farming.

Furthermore, the presence of cattle on the farm in terms of flock status was calculated to increase the odds ratio for BDV by 1.57 times compared to the absence of cattle (p -value:0.03<0.05; OR:1.57; 95% CI:1.03-2.42). Likewise, the presence of cattle and goats on the farm was calculated to increase the odds ratio for BDV by 1.67 times compared to sheep farms alone (p -value:0.01<0.05; OR:1.67; 95% CI: 1.09-2.56). This can be explained by the presence of

pestivirus transmission cases from cattle to sheep or from sheep to cattle, as has been reported in previous studies (McCullough et al., 1987; Paton et al., 1995; Lindberg et al., 1999; Krametter-Froetscher et al., 2007; Rosamilia et al., 2013; Kaiser et al., 2017; Şevik, 2020).

CONCLUSIONS

As a result, the prevalence of BDV in sheep may be an important risk factor for pestiviral infection of other species in the region, cattle that share common pastures with sheep. When seropositivity and PI rate of BDV infection are evaluated together, the removal of PI animals from the flock, taking and implementing biosecurity measures, ensuring the protection and control of the disease will be important for the livestock sector. Further studies aimed at isolating the BDV strains circulating in Turkey will help to understand the molecular epidemiology and dynamics of the pestivirus infections in the country better.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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