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Efficacy of a lyophilized combined Rift Valley Fever and Pest des Petits Ruminants vaccine

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Abstract
Background: Rift Valley Fever (RVF) and Pest des Petits Ruminants (PPR) are two viral diseases that pose a significant threat to sheep populations, leading to substantial financial losses.
Methods: The current study aimed to make a vaccine that contains both the inactivated RVF and the live attenuated PPR viruses in a freeze-dried form. This vaccine aimed to provide sheep with protection against both diseases with a single injection. It was made with the ZH501 strain of the RVF virus, which has a titer of $10^{7.5}$ $TCID_{50}$/ml, and the Nigerian PPR virus (N75/1), which was grown on the VERO cell line. The vaccine underwent quality control assessments. To make a lyophilized vaccine, inactivated RVF and attenuated PPR viruses were mixed and stabilizers of 1% and 0.5% carbomers were added.
Results: Evidence has shown that the vaccine was secure and free from foreign impurities such as mycoplasma, fungus, and anaerobic and aerobic bacteria. Furthermore, it does not have any detrimental consequences for unvaccinated sheep in close proximity. The vaccine demonstrated efficacy for over 6 months since the vaccine formulated with 0.5% carbomer established a sufficient level of protective immunity prior to the administration of the vaccine formulated with 1% carbomer.
Conclusion: It is advisable to utilize the vaccine containing 0.5% carbomer as a stabilizer, as it has been shown to rapidly achieve a protective level against both diseases. This approach allows for the administration of a single immunization, thereby saving time, effort, and money.
Keywords: Rift valley fever; peste des petits ruminants; lyophilization; carbomer; combined vaccine

1. Introduction

The sheep industry is a key contributor to the overall economy. Sheep are susceptible to various perilous diseases, particularly those caused by viruses, which have a significant impact on their population. Infectious animal diseases pose a significant threat to agriculture, with the potential to cause severe economic consequences for both industrialized and developing nations at local, regional, and global levels. Rift Valley Fever (RVF) and Peste des Petits Ruminants (PPR) are two viral illnesses that specifically target sheep and result in significant financial losses. RVF is primarily a viral hemorrhagic disease that mainly affects domestic animals, although it can occasionally afflict people as well. Outbreaks of RVF can result in substantial economic losses. The disease was initially identified in 1977, following a sudden and widespread outbreak in Egypt. In September 2000, the virus extended its presence to Saudi Arabia and Yemen, prompting concerns about its potential spread to additional areas in Europe and Asia (WHO, 2017). The condition mostly affects bovines, leading to premature parturition. RVF outbreaks called epizootics make it more likely that animals will get the disease and then infect people, which could lead to human RVF epidemics (CDC, 2020).

RVF virus (RVFV) was identified by the World Health Organization (WHO) in 2017 as one of the top ten viruses that pose a high risk of causing widespread epidemics. Barr and Wertz (2005) and Amarasinghe et al. (2018) have identified the RVFV as a member of the Phlebovirus genus within the Bunyaviridae family. The RVFV has only one serotype, as stated by Besselaar et al. in 1991. In 2009, the OIE reported that the virus may have many distinct virulence levels.

RVF has a sporadic pattern of occurrence, appearing every 5–25 years in animal populations that are not immune to the virus. This frequency is typically associated with specific seasons and increased rainfall (OIE, 2013). RVF was seen as a highly significant resurfacing disease, with outbreaks occurring following weather conditions that facilitate an increase in mosquito numbers. Maluleke et al. (2019) stated that RVF outbreaks occur in a cyclical pattern, often recurring every 10–15 years The expansion of RVF across the world in recent decades is a major concern (Nanyingi et al., 2015; Hatchett and Lurie, 2019). The main approach employed by current disease management programs to reduce the risk to humans is to control the spread of viruses within animal populations (Métras et al., 2020). The RVF vaccine inactivated with binary ethyleneimine was found to be safer and more

effective for vaccinating vulnerable animals compared to the formalin-inactivated vaccine (Eman, 1995).

PPR is an acute viral infection that primarily affects sheep, goats, and cattle. However, it can also have subclinical manifestations in pigs, camels, and other species. The causative virus belongs to the genus Morbillivirus and is closely associated with other viruses in the same family, including canine distemper and Rinderpest viruses (Amarasinghe et al., 2019). Parida et al. (2019) state that the causative virus is very contagious and can be transmitted by direct contact with the secretions of infected animals, as well as through contact with contaminated objects.

There is only one type of PPRV, but the sequencing of the nucleoprotein (N) and fusion protein (F) genes has shown that there are four different lineages (Misinzo et al., 2011). Asil et al. (2019) have reported that the sickness has been observed in a small number of animal species, including Dorcas's gazelles (Gazella dorcas). The morbidity rate, which refers to the proportion of individuals affected by a disease, ranges from 90% to 100%. The fatality rate, which refers to the proportion of individuals who die from the disease, ranges from 50% to 100%. These statistics are based on data from the OIE in 2009. Goats that are infected with PPR have also been associated with a significant increase in the rate of abortion (Abubakar et al., 2008). The presence of PPR has a substantial economic impact, as it acts as a considerable obstacle to the production of small ruminants in Africa (Ugochukwu et al., 2019). The initial outbreak of Peste des Petits Ruminants (PPR) in Egypt was documented in January 1987 (Ikram et al., 1988). According to the studies conducted by Mouaz et al. (1995), Abd El-Hakim (2006), Abd El-Rahim et al. (2010), Soltan and Abd-Eldaim (2014), Safwat (2015), Mahmoud et al. (2017), and Ahmed et al. (2021), PPR reemerged in a limited number of Egyptian governorates. A widely recognized approach for addressing this particular sickness is the PPR homologous live attenuated immunization (Khodeir and Mouaz, 1998; OIE, 1998). Mouaz et al. (1998) advocate for the combined use of RVF and PPR vaccines, which results in time, effort, and cost savings compared to individual treatments.

The objective of this study was to develop a safe and potent RVF and PPR combined for sheep aiming to protect them against both illnesses with a single vaccination, reducing stress on the animals and saving time, effort, and vaccination costs.

2. Materials and methods

Ethical approval

The Veterinary Serum and Vaccine Research Institute's Animal Ethics Committee approved this work (VSVRI). Every experiment conforms to the VSVRI guidelines for research with animals.

2.1. Viruses

The ZH501 strain of RVF virus with a titer of 107.5 TCID50/ml propagated on the VERO cell line was supplied by the Rift Valley Fever Vaccine Research Department (RVFVRD), Veterinary Serum and Vaccine Research Institute (VSVRI), Abasia, Cairo. The Nigerian PPR virus (N75/1) was attenuated through 6 passages on lamb kidney cell culture followed by 77 passages on VERO cells (AU-PANVAC) representing the master seed of the PPR virus. It was maintained and supplied by the Rinderpest Vaccine Research Department (RVRD), VSVRI. The two viruses were employed in serological testing as well as vaccine production.

2.2. Cell culture

African green monkey kidney (Vero) cell culture, obtained from the VSVRI refers to the process of growing and maintaining cells in a controlled environment outside of their natural setting. The cells were cultured in Minimum Essential Medium (MEM) supplemented with Earle's salts and 10% newborn calf serum at a temperature of 37 °C. The cell culture medium was supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin sulfate, and 25 IU/L mycostatin from Gibco Laboratories in New Zealand. These cells were utilized to propagate the two viruses in order to produce vaccines and conduct serological tests.

2.3. Vaccines

Single inactivated RVF and single attenuated PPR vaccines were supplied by VSVRI and used for the vaccination of experimental sheep in comparison to the potency of the prepared combined RVF and PPR vaccine

2.4. Preparation of the combined RVF and PPR vaccine

2.4.1. Preparation of inactivated RVF virus

The ZH501 RVFV strain with a titer of 107.5 TCID50 / ml was inactivated using BEI in accordance with Eman (1995). Verifying the process of inactivation to ensure total inactivation of the viral suspension, a validated inactivation control method was employed as follows: in cell culture, according to Ayoub and Allam (1981), complete virus inactivation was verified through two passages of the inactivated virus in cell culture showing no CPE. In baby mice, a further method of verifying virus inactivation involved the inoculation of the inactivated virus intracerebrally into young mice (Eman, 1995) revealing no clinical signs or deaths.

2.4.2. Preparation of attenuated Nigerian PPR virus (N75/1)

Suspension carried out according to Khodeir and Mouaz (1998) where each dose is 102.5 $TCID_{50}$/sheep.

2.4.3. Combined RVF and PPR lyophilized vaccine

Two equal volumes of the inactivated RVF and attenuated PPR viruses were mixed and prepared to be subjected to the lyophilization process in two formulae as follows: Fornula-1 using 1% Carbomer with 2% peptone as a stabilizer and Formula-2 using 0.5% Carbomer as a stabilizer. The used carbomer is Carbopol® 940 NF (Lubrizol®) polymer. It was diluted to 0.5% and 1% by the hot DDH2O and autoclaved at 121°C/20min and then stored at 4°C and calibrated to pH 7.3 before use. Each vaccine formula was mixed with an equal volume of sterile and chilled stabilizer to prepare the two formulae.

The two formulae were dispensed in neutral vials glass (2.5 ml/vial) and covered with a semipermeable rubber stopper then subjected to the lyophilization process on a Teflon lyophilizing apparatus. The inner diameter of the glass vial measured 1.9 cm. In order to achieve rapid freezing, the vials were placed on the freeze dryer's shelf, which had been previously cooled to -60°C (Zhang and Wang, 2007). Following two hours of chilling, the initial drying started. According to Zhou et al. (2007), the vacuum was maintained at 10 Pa, and the shelf temperature was fixed at -32°C. The first round of drying took place over 16 hours. After that, the shelf was heated to 20°C for six hours at a rate of 0.2°C per minute. Following freeze-drying, the vials were sealed and stored for two hours at room temperature (Shao-Zhi et al., 2010). They were then stored at -20°C until they were used in the experimental work.

2.5. Animals

Fifty Swiss Albino weaned mice (21-28 days old) were supplied by the Lab Animal Farm, VSVRI, and used for confirmation of complete RVFV inactivation and vaccine potency test. To assess the vaccine's immunogenicity and safety, twenty-five native breed male sheep, aged 6 to 8 months were employed. The animals were checked using a serum neutralization test and found to be free of RVF and PPR antibodies.

2.6. Quality control tests

2.6.1. Sterility test

It was completed in compliance with OIE (2019), Random samples of

the two prepared vaccine formulae were individually cultured on nutrient agar, Sabouraud agar, thioglycolate medium, and mycoplasma medium tubes.

2.6.2. Safety test

Randomly selected 5 vials of each of the final prepared combined RVF/PPR vaccine formulas were reconstituted in sterile normal saline to inject three sheep subcutaneously. Meanwhile, three other sheep are retained without any injection as control and housed in close proximity to the inoculated sheep for the next three weeks receiving balanced rations and adequate water under hygienic measures. During this phase, they undergo daily clinical examinations with recorded rectal temperature. If the vaccination does not cause any aberrant clinical symptoms and there is no indication of contact transmission of the vaccine virus, it is deemed safe.

2.6.3. RVF potency test in mice

The vaccine was diluted in fivefold dilution in appropriate conditions, ranging from 1:1 to 1:625. Five mice groups (8 mice/group) were used where each group was used for each dilution and each mouse received two doses of 0.2 ml of the vaccine inoculated I/P, on a week interval. Seven days following the second injection, 0.1 ml of the RVF virus, which contained 103 MIPLD50 / ml, was given intraperitoneally to all animals as a challenge under restricted hygienic measures in an insect-proof animal house. This was on top of the other two mouse groups, one of which received a challenge virus as a positive control and the other was maintained as a non-challenged, non-vaccinated negative control. The accepted level is below 0.02 (OIE, 2012). For 21 days, all mice groups were under observation, and every day's fatalities were noted. The ED50%/ml was computed using Reed and Muench (1938) equation. Deaths that transpired on the first day were regarded as non-specific.

Evaluation of the humeral immune response of sheep to the prepared combined RVF and PPR vaccine in comparison to their response to the single RVF and PPR vaccine was carried out through the following steps including 5 sheep groups (4 sheep/group): Group-1 Four sheep received a shot of the prepared combined RVF and PPR lyophilized vaccine formula (1) 2 ml inoculated subcutaneously (Samir et al., 1999). In group-2, four sheep received a shot of the prepared combined RVF and PPR lyophilized vaccine formula (2) 2 ml inoculated subcutaneously (Samir et al., 1999). Group-3 Four sheep received a shot of the inactivated RVF vaccine in a dose containing 107.5 TCID50 of the inactivated virus/animal 1ml inoculated subcutaneously (Eman, 1995). In group-4, four sheep received a shot of the single attenuated PPR vaccine in a dose of 102.5 TCID50/animal 1ml inoculated subcutaneously (Khodeir and Mouaz, 1998). Group-5 Three sheep were kept without inoculation as control.

Serum samples were obtained from all sheep groups on 0, 7. 14, 21, and 28 days then every month up to 6 months post-vaccination and subjected to monitoring of the exhibited antibody titers by SNT and indirect ELISA.

2.7. Serological test

2.7.1. Serum neutralization test (SNT)

According to Walker (2000), the test's goal was to find the specific antibodies that could neutralize both RVFV and PPRV. RVF and PPR antibody titers in vaccinated sheep were checked by using the micro-technique method of SNT formed by Ferreira (1976) on flat-bottom tissue culture microtiter plates on Vero cell culture. According to Singh et al. (1967), the endpoint of RVF/PPR neutralizing antibody titers was determined as the reciprocal of the last serum dilution that neutralized and inhibited the CPE of 100TCID50 of the used virus. According to Khodeir and Mouaz (1998) and OIE (2013), a positive titer of PPR serum-neutralizing antibody ≥8 is considered to be protective. It was carried out according to the OIE Manual (1996) where it was recommended that the positive titer of RVF neutralizing antibodies should not be less than >40 (WHO, 1983, OIE Manual, 1996 and Arwa et al., 2007).

2.7.2. Indirect Enzyme Linked Immune Sorbent Assay (ELISA)

An indirect ELISA assay was carried out following the direction of OIE (2016) for monitoring of RVF and PPR antibody titers as a confirmatory test.

3. Results

The outcomes attained showed that the prepared lyophilized combined RVF and PPR vaccine is devoid of external pollutants and safe inducing no post-vaccination local or systemic abnormal clinical signs in inoculated sheep (Table 1). Table (2) presents the findings of SNT, which show that the PPR antibody titer in immunized sheep consistently increased on vaccination with the combined RVF and PPR lyophilized vaccine with Carbomer 1% as a stabilizer (group-1) and in sheep vaccinated with a combined RVF and PPR lyophilized vaccine with Carbomer 0.5% as a stabilizer (group-2), the antibody titer reached its highest level (128) by the 4^{th} and 3^{rd} week post-vaccination, respectively, while those vaccinated with the single PPR vaccine exhibited their highest titer (64) by the second month. In contrast, the sheep that were not vaccinated remained negative for the antibodies. Table (3) demonstrates PPR mean values ELISA optical density indices which came in parallel to and confirmed the SNT results. Table (4) presents the results of SNT, indicating that the average RVF neutralizing antibody titer in the sera of sheep in group-1 (vaccinated with vaccine formula-1 containing Carbomer 1%) began to increase from the first week after vaccination and reached a protective level by the fourth week. In contrast, sheep in group-2 (vaccinated with formula-2 vaccine containing Carbomer 0.5%) showed an increase in neutralizing antibody titer from the first week after vaccination and reached the protective level by the third week. The acquired antibody titers remained at consistent values for up to 6 months following immunization over the experimental period. The tabulated results in Table (5) demonstrate the RVF mean values of ELISA optical density indices came in the same manner as SNT results.

Table 1. Quality control of combined lyophilized RVF and PPR vaccine

Tested parameter	RVF		PPR	
	Formula 1	Formula 2	Formula 1	Formula 2
Sterility	Free from foreign contaminants			
Safety in sheep	Safe			
Potency *	0.0019	0.0014	potent	

* The accepted level is below 0.02. Formula 1: combined RVF and PPR vaccine with Carbomer 1% as a stabilizer. Formula 2: the prepared combined RVF and PPR vaccine with Carbomer 0.5% as a stabilizer

Table 2. PPR serum neutralizing antibody titer in experimentally vaccinated sheep groups

Sheep groups	Mean PPR serum neutralizing antibody titer*									
	0	1WPV**	2WPV	3WPV	4WPV	2MPV***	3MPV	4MPV	5MPV	6MPV
Group-1	0	4	16	64	128	128	128	128	128	128
Group-2	0	4	32	128	128	128	128	128	128	128
Group-4	0	4	8	16	32	64	64	64	64	64
Group-5	0	0	0	0	0	0	0	0	0	0

*PPR antibody titer = the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of PPR virus; PPR serum neutralizing antibody titer ≥8 is considered protective. (OIE, 3013 and Santhosh et al., 2013), **WPV= Week Post Vaccination, ***MPV= month post vaccination. Group-1: vaccinated with the prepared combined RVF and PPR lyophilized vaccine Formula (1) with Carbomer 1% as a stabilizer. Group-2: vaccinated with the prepared combined RVF and PPR lyophilized vaccine Formula (2) with Carbomer 0.5% as a stabilizer. Group-4: vaccinated with a single PPR vaccine. Group-5: Unvaccinated control

Table 3. PPR Mean values of ELISA optical density indices

Sheep groups	PPR Mean values ELISA optical density indices									
	0	1WPV*	2WPV	3WPV	4WPV	2MPV**	3MPV	4MPV	5MPV	6MPV
Group-1	0.024	0.085	0.207	0.245	0.321	0.314	0.311	0.317	0.322	0.324
Group-2	0.057	0.097	0.282	0.341	0.339	0.340	0.337	0.341	0.340	0.341
Group-4	0.049	0.074	0.159	0.195	0.271	0.297	0.294	0.289	0.294	0.298
Group-5	0.035	0.045	0.041	0.039	0.038	0.044	0.042	0.039	0.041	0.038

*WPV= Week Post Vaccination, **MPV= month post vaccination. Cut-off value = 0.157

Table 4. RVF serum neutralizing antibody titer in experimentally vaccinated sheep groups

Sheep groups	Mean RVF serum neutralizing antibody titer*									
	0	1WPV**	2WPV	3WPV	4WPV	2MPV***	3MPV	4MPV	5MPV	6MPV
Group-1	0	8	16	32	64	128	128	128	128	128
Group-2	0	16	32	64	128	128	128	128	128	128
Group-3	0	16	32	64	64	128	128	64	64	64
Group-5	0	0	0	0	0	0	0	0	0	0

*RVF neutralizing antibody titer = the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of the virus. The protective level of RVF-neutralizing antibody titer is >40. **WPV= Week Post Vaccination, ***MPV= month post vaccination.

Table 5. RVF mean values of ELISA optical density indices

Sheep groups	RVF Mean values ELISA optical density indices									
	0	1WPV*	2WPV	3WPV	4WPV	2MPV**	3MPV	4MPV	5MPV	6MPV
Group-1	0.029	0.095	0.119	0.159	0.192	0.296	0.291	0.289	0.292	0.287
Group-2	0.048	0.107	0.145	0.194	0.314	0.311	0.307	0.313	0.309	0.312
Group-3	0.036	0.091	0.125	0.187	0.243	0.299	0.296	0.287	0.291	0.293
Group-5	0.039	0.042	0.044	0.039	0.037	0.041	0.045	0.039	0.043	0.037

Cut-off value = 0.179. *WPV= Week Post Vaccination, **MPV= month post-vaccination

4. Discussion

RVF and PPR, which are highly contagious diseases, have a significant economic impact on the sheep population among small ruminants. The epizootic scenario highlights the necessity of creating a very efficient method for specific protection against both RVF and PPR infections. A composite vaccine has been developed to provide ongoing protection for sheep against RVF and PPR. The vaccine was prepared with the inactivated RVFV, the ZH501 strain, and the attenuated PPRV (Nigeria 75/1 PPR strain). It was manufactured in a lyophilized form with two different concentrations, 1% and 0.5%, using carbomer as a stabilizer. Such vaccines are advisable to have combined vaccines available in order to reduce disease transmission, decrease global immunization expenses, improve acceptance, and streamline their execution (Francis, 1999). The intriguing findings suggest the possibility of combining RVF and PPR vaccinations, which would streamline the process and reduce the resources required for separate processes (Mouaz et al., 1998). Following the OIE's (2019) guidelines, tests on the combined RVF and PPR lyophilized vaccine showed that it is free of anaerobic and aerobic bacteria, mycoplasma, and fungi.

The vaccine safety assessment was conducted in sheep through subcutaneous vaccinations using 100 field doses of a combined vaccine, following the methodology established by Madhusudan et al. (2006). The results indicated that both the vaccinated and unvaccinated control sheep maintained body temperatures within the normal range (38.5–39.8 °C). Furthermore, the clinical condition of the vaccinated sheep remained within normal limits for two weeks, as presented in Tables 1 and 2. Furthermore, it is worth noting that both the vaccinated and control sheep exhibited no alterations in their appetite or behavior for three weeks following vaccination, indicating their sustained health (Mouaz et al., 1998). Additionally, the PPR vaccination is considered to be safe and does not have a substantial negative impact on the host's immune system (Rajak et al., 2005). The results showed that the lyophilized RVF and PPR vaccines were safe and did not cause any abnormal local or systemic clinical signs in the vaccinated sheep.

The potency of the combined vaccine against the RVFV was determined using the ED_{50}/mL method in mice (OIE, 2012). In the serum neutralization test (SNT), it was found that the level of antibodies against PPR steadily rose in sheep that had been immunized with the RVF and PPR lyophilized vaccine with 1% carbomer as a stabilizer (group 1) and in those vaccinated with a lyophilized vaccine containing both RVF and PPR and carbomer 0.5% as a stabilizer (group 2) showing a peak antibody titer of 128 by 4 and 3 weeks after vaccination. In contrast, unvaccinated sheep did not show any antibodies in their sera. Keeping an eye on the levels of PPR antibodies in vaccinated sheep showed that the SNT results showed that these sheep had protective levels of PPR serum neutralizing. These titers ranged from 8–32 by the 2nd week after vaccination, to 16 in group 1 (vaccinated with formula 1 of the combined RVF and PPR vaccine), 32 in group 2 (vaccinated with formula 2 of the combined vaccine), and 8 in group 4 (vaccinated with the single PPR vaccine). These titers are deemed to be within the recommended protective levels, as indicated by Khodeir and Mouaz (1998). According to the OIE (2013) and Santhosh et al. (2013), sheep or goats with a PPR serum-neutralizing antibody titer of at least 10 three weeks after vaccination are considered to have immune-stimulating properties. By the fourth week after vaccination, the antibody titers in the three vaccinated sheep groups had reached their highest level (128). These levels remained unchanged for up to 6 months which was the duration of the experiment. This result is similar to what was recorded by Khodeir and Mouaz (1998) and Mouaz et al. (1998). They also found an increase in antibody titers after the PPR vaccination with the highest levels happening on day 28. This suggests that the immunity provided by the vaccine lasts for a longer time.

The study examined the humeral immune response to RVF induced by the combined vaccine generated by SNT. It was found that the vaccinated sheep exhibited protective levels of RVF serum-neutralizing antibody titers. These antibody indices remained unchanged for up to 6 months after vaccination, which is consistent with the findings reported by Mouaz et al. (1998). The peaks of antibody levels were seen to be 128 in both group-1 and group-2 sheep by the 8th and 4th weeks post-vaccination, respectively. In contrast, the sheep that were not vaccinated did not show any detectable antibodies. According to WHO (1983), the OIE Manual (1996), and Arwa et al. (2007), the level of RVF-neutralizing antibodies should be at least >40 for stopping the RVFV.

Conclusion

Our present results indicate that the prepared lyophilized combined RVF/PPR vaccine is safe and potent without antagonizing the effect between the two viral contents on the sheep immune response. Such a vaccine has the ability to save cost- time and effort and can serve as a replacement for individual immunizations against PPR and RVF. It is advisable to use a vaccination containing 0.5% carbomer as a stabilizer in order to confer sheep with robust and enduring protection.

Conflict of interest

The authors reported the absence of any possible conflicts of interest.

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Authors' contribution

Every author offered feedback on earlier drafts of the manuscript and helped with the planning and design of the study. The final manuscript was read and approved by them.

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