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Randomised field trial to evaluate serological response after foot-and-mouth disease vaccination in Turkey

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ABSTRACT

Despite years of biannual mass vaccination of cattle, foot-and-mouth disease (FMD) remains uncontrolled in Anatolian Turkey. To evaluate protection after mass vaccination we measured post-vaccination antibodies in a cohort of cattle (serotypes O, A and Asia-1). To obtain results reflecting typical field protection, participants were randomly sampled from across Central and Western Turkey after routine vaccination. Giving two-doses one month apart is recommended when cattle are first vaccinated against FMD. However, due to cost and logistics, this is not routinely performed in Turkey, and elsewhere. Nested within the cohort, we conducted a randomised trial comparing post-vaccination antibodies after a single-dose versus a two-dose primary vaccination course.

Four to five months after vaccination, only a third of single-vaccinated cattle had antibody levels above a threshold associated with protection. A third never reached this threshold, even at peak response one month after vaccination. It was not until animals had received three vaccine doses in their lifetime, vaccinating every six months, that most (64% to 86% depending on serotype) maintained antibody levels above this threshold. By this time cattle would be >20 months old with almost half the population below this age. Consequently, many vaccinated animals will be unprotected for much of the year. Compared to a single-dose, a primary vaccination course of two-doses greatly improved the level and duration of immunity. We concluded that the FMD vaccination programme in Anatolian Turkey did not produce the high levels of immunity required. Higher potency vaccines are now used throughout Turkey, with a two-dose primary course in certain areas.

Monitoring post-vaccination serology is an important component of evaluation for FMD vaccination programmes. However, consideration must be given to which antigens are present in the test, the vaccine and the field virus. Differences between these antigens affect the relationship between antibody titre and protection.

1. Introduction

As the duration of FMD vaccine protection is short lived, animals require regular revaccination. In Turkey cattle are routinely vaccinated twice a year [1–4]. It is recommended that after initial vaccination at two months of age, cattle receive a second dose one month later. However, as mass vaccination is costly [5], some countries, including Turkey, use a single-dose primary vaccination course.

Much is known about immunity after a single dose of high potency vaccine used to control outbreaks in free countries [6–10]. However, requirements in this setting differ to the sustained protection required in endemic countries where standard potency ($\geq$3PD$_{50}$) FMD vaccines are typically used (PD$_{50}$ $\equiv$ 50% protective dose). Limited protection after a single dose of $\geq$3PD$_{50}$ FMD vaccine is not uncommon [4].

FMD structural protein (SP) antibody levels are strongly correlated with protection [11–18]. In this prospective field study, we assessed post-vaccination SP antibody levels in a cohort of cattle, vaccinated within the Turkish FMD vaccination programme, the

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objective being to evaluate vaccine protection in the population at large. A randomised trial, with two parallel arms was nested within the cohort to assess the effect of administering two vaccine doses approximately one to two months apart as opposed to a single dose.

2. Materials and methods

2.1. Study design and sampling

2.1.1. Background and village selection

Households were selected from an FMD sero-prevalence survey conducted in Anatolian Turkey in September–November 2012 ("autumn"). We present results of the prospective study only and not the sero-prevalence survey. In the survey, cattle were randomly sampled from each of 1027 villages, randomly selected across Turkey, stratified by region, using the national livestock database as a sampling frame.

Villages in Central and Western Anatolia conducted routine FMD vaccination immediately after sampling. Prospective study eligibility was restricted to villages that vaccinated one to two months before December 2012 ("winter") for which serology results were available. From these 37 villages, four were inaccessible due to heavy snow, one could not be sampled as cattle were at grazing and a further nine villages were excluded due to inadequate vaccination records. This left 98 households in 23 villages, from eight provinces, included in this prospective study (see Fig. 1).

2.1.2. Sampling

Each household was visited in December 2012 ("winter") and again in late February or early March 2013 ("spring"). During December, all cattle <24 months old present at enrolled households were sampled, including those not sampled in the autumn sero-prevalence survey. Those that tested positive for non-structural protein (NSP) antibodies at autumn sampling, indicating prior infection, were excluded. Vaccines used were purified for NSP proteins, so, unlike infection, vaccination rarely leads to NSP sero-positivity. This differs from SP antibodies which are produced after infection or vaccination. Of 736 animals sampled during winter, 355 had been sampled in autumn 2012. Animals were identified by unique ear-tag numbers, something all Turkish cattle should have.

2.1.3. Booster allocation

At winter sampling, half the cattle in each household were given an additional dose of Şap institute trivalent FMD vaccine. Animals within a household were divided into two equally sized groups, balanced in age and prior FMD vaccination status. One group was then randomly selected to receive an additional dose of vaccine if the last ear-tag digit of the first animal selected was <5; the other group received no additional vaccination. Animals under two months of age were not vaccinated. Animals not previously vaccinated were randomised separately with one-in-four selected for vaccination.

2.1.4. Additional information

Farmers and investigators were present during vaccination and were not blinded. Outcomes were serological and those conducting the laboratory tests were blinded from the details of the animals being tested. Study data were only available to T.J.D.Knight-Jones. Animal housing and location remained unchanged throughout the study.

2.1.5. Vaccination and sampling procedures

During autumn, animals were sampled and vaccinated by state veterinary staff. Winter and spring sampling was conducted by T.J.D.Knight-Jones, A.N.Bulut and M.Alkan, when animals were briefly examined and blood sampled, with additional vaccination for selected animals. Animal and holding details were collected, including information on prior vaccination, disease, trading and husbandry. All animals were permanently housed during the study with turnout for grazing commencing shortly after final sampling.

2.1.6. Vaccines

The $\geq$3 PD_{50}, NSP purified Şap institute (Ankara, Turkey) trivalent FMD vaccine, contains strains O Panasia II (O Tur 07), A Iran-05 (A TUR 06) and Asia-1 Sindh-08 (Asia-1 TUR 11). Six different vaccine batches were used in autumn vaccination. A single batch was used within a province, with 2 ml injected intra-muscularly for each dose. For all winter vaccination a single batch was used.

2.1.7. Serology

Sera were tested for NSP antibodies (PrioCHECK FMDV NS ELISA-Prionics, Zurich, Switzerland). Sera were also assessed for SP antibodies for the vaccine serotypes using the liquid phase blocking ELISA (LPBE), supplied by The Pirbright Institute, UK. The strains of virus used to produce the ELISA antigens (O Manisa, A22 IRQ 24/64 and Asia-1 Shamir) could not be changed and were different to the strains used to produce the vaccine. These differences were antigenically significant, based on serological matching tests (WRLFMD, The Pirbright Institute).

Sera taken during autumn 2012 sampling were tested for SP antibodies using a single dilution of $1{:}10^2$. This titre is associated with approximately 70% clinical protection [19], assessed by the vaccine manufacturer and other published studies, the latter using a homologous test system [20,21]. Sera collected at winter and

Fig. 1. Map of Turkey showing the location of villages included in the study. As mass vaccination was not conducted in Eastern Turkey in autumn 2012 sampled villages come only from Central and Western Turkey.

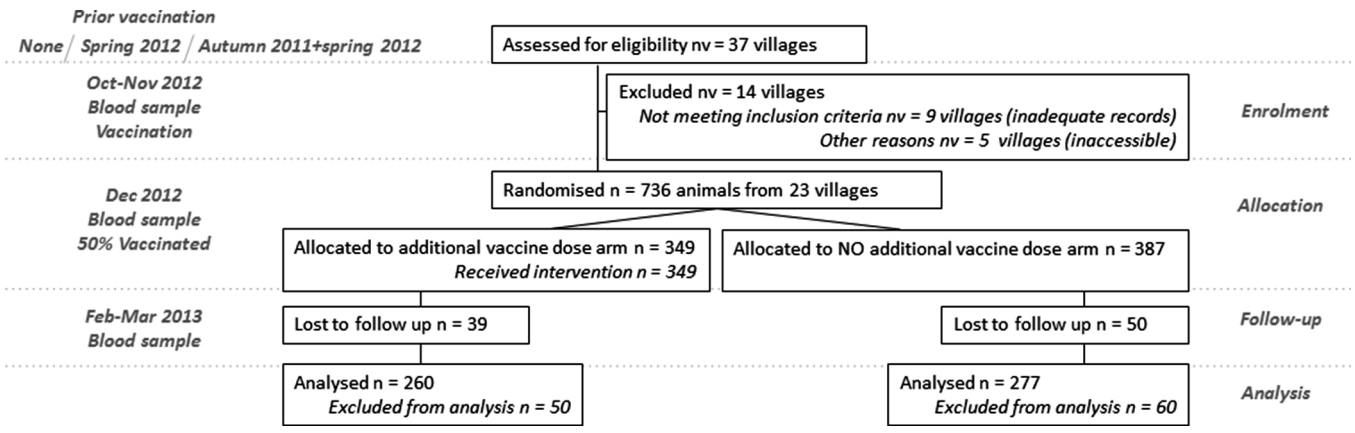

Fig. 2. Flow diagram and timeline, showing for each study group, the number of participants who were randomly assigned to receive the additional dose of vaccine that were ultimately analysed for the primary outcome.

spring sampling were titrated for SP antibodies at dilutions of 1:32, 1:45, 1:64, 1:96, 1:128, 1:192 and 1:256. NSP and LPBE testing were performed at the Şap institute, Turkey.

2.1.8. Virus neutralisation (VN) tests

To further investigate the significance of mismatch between the LPBE and vaccine antigens, VN tests were performed on a subset of 31 cattle from two nearby villages, first vaccinated in autumn 2012 by the same operator on the same day using the same vaccine batch. When sampled in spring, it was 109 days since the 18 single vaccinated cattle were last vaccinated and 76 days for the 13 cattle that received the two-dose primary course.

VN tests were performed at The Pirbright Institute (WRLFMD) using the vaccine strains, the LPBE strains and two current field strains of concern (O India-2001^{KAR-13}, A Iran-05^{SIS-10}). Dilutions used were 1 in: 16, 22, 32, 45, 64, 90, 128, 178, 256, 355, 512, 708, 1024 and 1413.

2.2. Data processing and analysis

2.2.1. SP serology

Samples positive for NSP antibodies, produced after prior infection and not vaccination, were excluded. For cattle first vaccinated in autumn 2012, those with prior positivity for SP antibodies were also excluded.

Differences in SP titres at winter and spring sampling were compared for animals with differing vaccination histories. SP titres were also compared for animals of different sex, province and breed. Comparisons were done using unpaired *t*-tests and boxplots.

Regression modelling was performed to support the univariable analysis and is included in the ESM. Data collection sheets, vaccine batch testing details and a power calculation are also included in the ESM.

2.2.2. Virus neutralisation

A "70% protective" threshold VN titre was derived from Barnett et al. (2003) [11], i.e. 1:$10^{1.71}$, 1:$10^{1.63}$ and 1:$10^{1.89}$ for serotypes O, A and Asia-1 respectively; with 1:10^{2} for LPBE. The proportion above the protection threshold for LPBE and VN results were compared using tests for paired data with confidence intervals produced by bootstrapping with 1000 samples.

Analysis was done in R [22] with the lme4 package [23].

3. Results

Except for one commercial dairy farm, all holdings were traditional small-holdings within villages, where most households keep a few cattle. From the 736 cattle initially randomised, only 647 animals (88%) were re-sampled in spring. Loss to follow-up consisted of 5 (1%) cattle inadequately sampled and 84 (11%) not available, mostly sold to provide income, although 23 had lost ear tags and could not be identified.

3.1. Sample population

Once NSP positive cattle and the few left unvaccinated in autumn were excluded, 537 cattle remained, with 260 in the additional winter vaccine group and 277 in the control group. From these cattle, 384 were unvaccinated prior to autumn and remained SP negative at autumn sampling; 189 in the additional vaccine group and 195 controls (Fig. 2).

3.1.1. Sampled population description

Treatment groups were similar with respect to age ($p \equiv 0.13$; Fig. S1) and prior vaccination. Mean number of doses prior to autumn 2012 vaccination was 0.25 and 0.33 in the intervention and control groups respectively ($p \equiv 0.09$). Mean time since last vaccination when sampled in spring was 72 days in the intervention group, last vaccinated during winter, and 115 days in the control arm, last vaccinated in autumn.

3.2. Post-vaccination SP serology

The mean LPBE SP titre at spring sampling for serotype O was 173 (or $10^{2.23}$) (95% CI: 162–184) in the intervention group and 82 (or $10^{1.91}$) (95% CI: 71–92) in those not vaccinated during winter (difference ≡ 91, 95% CI: 77–106, $p < 0.0001$). For serotype A, mean titre was 120 (or $10^{2.08}$) (95% CI: 109–131) and 52 (or $10^{1.72}$) (95% CI: 43–61) in the intervention and control group (difference ≡ 67, 95% CI: 54–82, $p < 0.0001$) and in the same order mean titre was 167 (or $10^{2.22}$) (95% CI: 157–179) and 83 (or $10^{1.91}$) (95% CI: 74–92) for serotype Asia-1 (difference = 84, 95% CI: 70–98, $p < 0.0001$). Even after adjusting for differences in time since last vaccination, the effect of the two-dose primary course was sizable (see regression modelling, ESM).

Table 1 shows that with no prior vaccination, approximately one month after autumn vaccination one third had SP titres below 1:10^{2} for serotypes O and Asia-1. Two-thirds had a titre below 1:10^{2} for serotype A. Come spring, three-quarters of those that received the extra vaccine dose had titres over 1:10^{2} (serotypes O and Asia-1); for serotype A over half of this group had above threshold titres. For those not revaccinated in winter, by spring, two-thirds had a low titre (<1:10^{2}) for all serotypes. Those also vaccinated in spring 2012, six months prior to the study, had slightly higher titres; and

Table 1
Breakdown of post-vaccination FMD SP titres of cattle at spring 2013 sampling following autumn 2012 vaccination. Half of the cattle were selected at random to receive an additional dose of the trivalent FMD vaccine during winter 2012/2013, one to two months after the autumn 2012 dose. The proportion of animals with a titre $\geq 1{:}10^2$ is shown along with the group mean titre. N.B. Some cattle included in the winter sampling sero-converted for NSP antibodies by spring sampling and were excluded from the analysis. Spring sampling was conducted 114 days (mean) after autumn vaccination, 90% range: 101–133 days.

		Number of doses received prior to autumn 2012 vaccination[a]							
		0 prior doses			1 prior dose			2 prior doses	
Sample		Winter	Spring		Winter	Spring		Winter	Spring	
Additional winter vaccine		All cattle	No	Yes	All cattle	No	Yes	All cattle	No	Yes
Serotype O	**SP titre $\geq 10^2$**	278/429 (65%)	77/200 (38%)	164/202 (81%)	87/114 (76%)	30/63 (48%)	46/51 (90%)	16/23 (70%)	12/14 (86%)	6/7 (86%)
	Relative risk[b] [95% CI]		*2.1 [1.8 to 2.5]*			*1.9 [1.4 to 2.5]*			*1 [0.2 to 5.6]*	
	Mean SP titre	126	74	170	141	88	183	160	157	176
	Difference [95% CI]	–	*96 [79 to 113]*		–	*95 [64 to 125]*		–	*18 [−60 to 97]*	
Serotype A	**SP titre $\geq 10^2$**	168/429 (39%)	52/200 (26%)	127/202 (63%)	57/114 (50%)	22/63 (35%)	39/51 (76%)	11/23 (48%)	9/14 (64%)	4/7 (57%)
	Relative risk [95% CI]		*2.4 [1.9 to 3.1]*			*2.2 [1.5 to 3.1]*			*0.9 [0.2 to 3.8]*	
	Mean SP titre	74	45	115	90	62	141	111	111	107
	Difference [95% CI]	–	*70 [54 to 86]*		–	*80 [49 to 110]*		–	*−3 [−110 to 103]*	
Serotype Asia-1	**SP titre $\geq 10^2$**	304/429 (71%)	84/200 (42%)	162/202 (80%)	88/114 (77%)	33/63 (52%)	43/51 (84%)	16/23 (70%)	12/14 (86%)	6/7 (86%)
	Relative risk [95% CI]		*1.9 [1.6 to 2.3]*			*1.6 [1.2 to 2.1]*			*1 [0.2 to 5.6]*	
	Mean SP titre	138	77	167	145	91	168	163	136	169
	Difference [95% CI]	–	*90 [74 to 117]*		–	*−76 [46 to 107]*		–	*33 [−62 to 129]*	

[a] All animals were vaccinated in autumn 2012.
[b] Relative risk is the ratio of the proportion positive in the treatment group compared to the proportion positive in the control group.

antibody levels of those vaccinated in both autumn 2011 and spring 2012 were higher still, reducing the benefit of additional winter vaccination (Table 1, S1, S2 and Fig. 3).

As the two-dose primary course concerns cattle previously unvaccinated, the effect of the additional winter dose on cattle vaccinated before autumn 2012 was less relevant. However, analysis of all cattle, without exclusion, does not depend upon the accurate recollection of vaccine history.

3.3. Univariable analysis

3.3.1. Inter-vaccination interval

Previously unvaccinated cattle with a primary course inter-vaccination interval of 14–30 days had a mean serotype O SP titre of 140, compared to 61 in autumn-only vaccinated animals belonging to the same households with difference in means of 79 [95% CI: 61–140]. If the interval was 31–74 days, mean titres were 178 in autumn and winter vaccinated cattle and 74 in those vaccinated in autumn only with difference in means of 104 [95% CI: 84–124]. For serotype A with a 14–30 day interval, mean titres were 72 and 22 in the treatment and control group with difference in means of 50 [95% CI: 28–72]; with the 31–74 day interval means were 126 and 51 with difference in means of 75 [95% CI: 55–95]. For serotype Asia-1 with the 14–30 day interval means were 130 and 76 in the treatment and control group with difference in means of 76 [95% CI: 44–107], and with the 31–74 day interval, means were 177 and 82 with difference in means of 95 [95% CI: 77–114]. Titres were similar for inter-vaccination periods between 31 and 74 days (Fig. S5).

Further univariable analysis and regression modelling are included in the ESM.

3.4. Virus neutralisation tests

Titres were greater when assessed against the homologous vaccine virus than for the heterologous virus used to make the LPBE (Fig. 4). For serotypes O and A, VN against the heterologous viruses mirrored protection according to the LPBE, which uses equivalent antigens. However, for Asia-1, more animals had higher titres according to the LPBE than for VN, even when virus homologous to the vaccine was used in the VN (Table 2).

4. Discussion

The rapid decline in immunity post-vaccination left many cattle susceptible long before the next six-monthly round of vaccination. The majority of cattle required three doses of vaccine to develop and maintain adequate antibody levels when vaccinated every six months. However, an animal will be at least 20 months old before it will have received three doses. The two-dose primary course incurs additional costs. However, without it many more young animals were unprotected for much of the year. This may not be the case for higher potency vaccines [6,24]. An increased immune response with increased time (>30 days) between the two doses of a primary course has been observed before [1].

Since this study was performed, as well as changing to a two-dose primary course in certain areas, the routine vaccine has been changed to a $\geq 6PD_{50}$ vaccine throughout Turkey. With the $\geq 3PD_{50}$ FMD vaccine, at least three out of every five single vaccinated cattle were likely to be susceptible to FMD serotypes O and Asia-1 three to four months after vaccination. For serotype A, even more would be susceptible. A third or more never attain a protective titre. Short-lived and limited vaccine protection appears to have contributed to the high incidence of FMD in Turkey despite widespread vaccination.

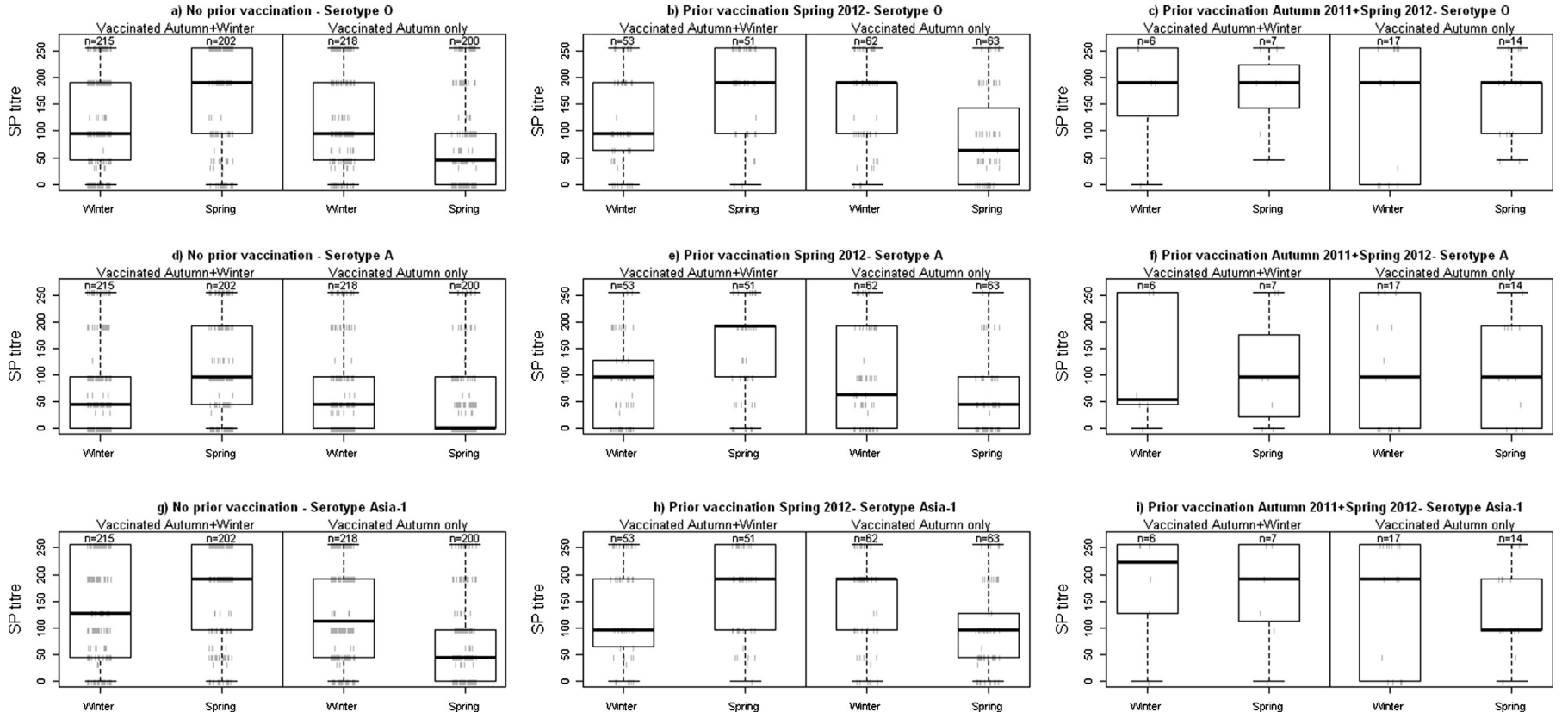

Fig. 3. Boxplots showing winter and spring SP titres for FMD serotypes O, A and Asia-1 from animals that did and did not receive the winter dose of vaccine. Shown separately for animals that had received differing numbers of vaccine doses prior to the study. Boxplots show the median (horizontal line), interquartile range (box) and range (whiskers). Group sample sizes are shown above each box and individual data points are marked as grey vertical dashes. Titres below the detection threshold were given a value of zero.

Table 2
Table showing agreement between protection levels estimated by LPBE and virus neutralisation (VN) using the virus homologous to the vaccine (V) and heterologous viruses including that used for the LPBE antigen (E). The estimated proportion with a VN titre over the 70% protection threshold is shown [11], this has been stratified according to LPBE titre. The median difference between the proportion protected (titre≥threshold) according to VN (V) and LPBE is shown *in italics with [2.5th and 97.5th percentiles]*. 70% protection thresholds of $1{:}10^{2}$ for LPBE and $1{:}10^{1.71}$, $1{:}10^{1.63}$ and $1{:}10^{1.89}$ for VN tests against serotypes O, A and Asia-1 respectively [11]. The minimum dilution for LPBE was $10^{1.5}$ (1:32).

	VN Virus	VN titre ≥70% protection threshold (%)				LPBE titre ≥10^2 (%)
		LPBE <$10^{1.5}$	LPBE >$10^{1.5}$ to –<10^2	LPBE ≥10^2	Total	
O	**(V) Panasia II (TUR 07)**	2/9 (22%)	9/11 (82%)	11/11 (100%)	22/31 (71%)	11/31 (35%)
	Difference [95% CI]	*22% [0-50%]*	*82% [55–100%]*	*0%*[a]	*36% [19–52%]*	
	(E) Manisa	0/9 (0%)	2/11 (18%)	9/11 (82%)	11/31 (35%)	
	India-2001^{KAR-13}	1/9 (11%)	1/11 (9%)	6/10 (60%)	8/30 (27%)	
A	**(V) Iran-05 (TUR 06)**	4/16 (25%)	5/7 (71%)	8/8 (100%)	17/31 (55%)	8/31 (26%)
	Difference [95% CI]	*25% [6–48%]*	*71% [33–100%]*	*0%*[a]	*29% [16–45%]*	
	(E) 22IRQ 24/64	0/16 (0%)	1/7 (14%)	5/8 (63%)	6/31 (19%)	
	Iran-05^{SIS-10}	1/16 (6%)	2/6 (33%)	3/8 (38%)	6/30 (20%)	
Asia-1	**(V) Sindh-08 (TUR 11)**	0/2 (0%)	2/16 (12.5%)	8/13 (62%)	10/31 (32%)	13/31 (42%)
	Difference [95% CI]	*0% [0–66%]*[a]	*12% [0–31%]*	*−39% [−67 to −13%]*	*−10% [−26 to −6%]*[b]	
	(E) Shamir	0/2 (0%)	0/16 (0%)	2/13 (15%)	2/31 (6%[c])	

[a] Bootstrap 95% CI of 0–0% as no variation in original results so binomial proportion used.
[b] −3% [−19 to −13%] difference in proportion over the 70% protection threshold if common O, A, Asia-1 VN threshold ($1{:}10^{1.67}$) used instead of Asia-1 specific VN threshold ($1{:}10^{1.89}$).
[c] 13% with titre ≥common O, A, Asia-1 70% protection threshold ($1{:}10^{1.67}$) which is lower than the Asia-1 only threshold [11]

Vaccines will provide better protection against challenge with a homologous virus than one antigenically different to the vaccine strain, as illustrated by the VN results. At spring sampling, for serotypes A and O only a third were above the antibody protection threshold by LPBE whereas half to two-thirds were above a protection threshold assessed by VN performed with the vaccine homologous virus. However, changing a vaccine strain is difficult and field viruses with limited vaccine match often arise. The low VN titres for the heterologous O India-2001^{KAR-13} and A Iran-05^{SIS-10} strains, suggest limited vaccine protection [4].

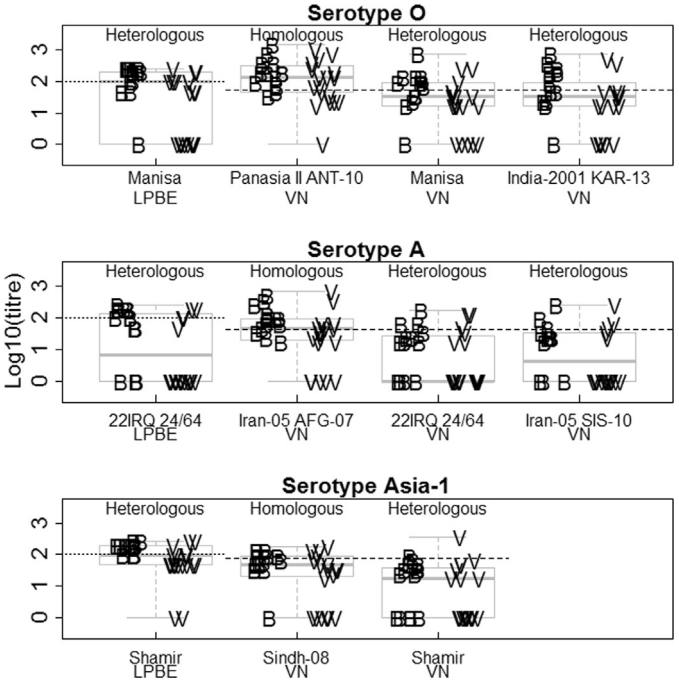

Fig. 4. Log_{10} (titre) of virus neutralisation tests against the homologous vaccine virus and the heterologous virus used in the LPBE test. Additional field strains of concern were also tested (O India-2001^{KAR-13} and A Iran-05^{SIS-10}). Results are shown for serotypes O, A and Asia-1 for sera collected in spring 2013 from 31 cattle first vaccinated in Autumn 2012. Original LPBE results are also included. Thresholds for ≈70% protection are indicated by the dashed lines. Results marked with a "B" are from animals given a second vaccine dose one month after autumn vaccination whereas those marked "V" have only been vaccinated in autumn 2012. Titres below the detection threshold were given a value of zero.

Contrary to what might be expected, protection against the Asia-1 serotype estimated by VN with the virus used to produce the vaccine was lower than for LPBE using antigen from a strain different to the vaccine strain. VN is less reproducible than LPBE and assessing protection by serology is never perfect [25]. Also, after batch release testing (batch 12_06, Fig. S3), the Asia-1 serotype may have degraded, although serotype O is typically more unstable. Degraded vaccine elicits more non-neutralising antibody that reacts in ELISA but not VN.

Field studies of the Asia-1 TUR 11 vaccine during outbreaks of the TUR11 virus found reasonable protection against clinical disease after a single dose with vaccine effectiveness of 69% (95% CI: 50–81%) [26]. Although vaccination reduced the risk, one-in-three vaccinated cattle still developed FMD [26]. This was similar to the proportion with an Asia-1 SP titre below the LPBE protection threshold ($1{:}10^{2}$) at winter sampling, also assessed one to two months post-vaccination.

When inferring FMD protection from antibody titre, the serological test used should be correlated with protection [19]. If a test antigen is used that differs to the one evaluated in the challenge study, the extent to which titre will reflect protection is more uncertain. Although variation in the reactivity of sera against different viral strains will reflect variation in the likelihood of protection [4], we are currently unable to quantify this variation with confidence. Quantification of protection requires virus challenge, either in the field or under controlled conditions, and for serological predictions, protection must be correlated with a specific assay.

Serological protection thresholds are typically derived from experimental potency tests where a high dose of challenge virus is injected into the tongue, 3–4 weeks after a single vaccine dose. The challenge virus is usually homologous to the vaccine and test strain. Protection in the field may differ, as virus challenge may be less intense but often more prolonged, and cattle have often been vaccinated many times with the last dose given 0–6 months prior to challenge. Although evaluation during field challenge is recommended [27], differences in SP titre between the vaccine groups in this study were large enough to expect sizable differences in susceptibility.

Routinely administered, the two-dose primary course would incur significant costs. This may be justifiable as protection in those <20 months age is greatly improved. This study assessed only cattle

vaccinated 1–4 times. There will be many unvaccinated cattle in the population at large, plus older cattle, vaccinated many times. Population immunity is complex and changes with population turnover, coverage and the proportion vaccinated multiple times. Further consideration requires a modelling approach.

5. Conclusion

When cattle in Turkey were vaccinated against FMD at six-monthly intervals, only after receiving three doses in their lifetime did most cattle maintain a high level of antibodies for more than three to four months. Thus, cattle under two years old, comprising 50% of the population [28], and many older cattle, appeared susceptible to FMD for half the year. A third never developed a satisfactory titre following a single vaccine dose.

Differences in vaccine and serological test antigens influenced titre, reflecting lower protection when challenged with a poorly matched field virus. However, in order to predict protection from serology, the relationship between titre, measured with a particular test, and protection, against a particular strain, should be quantified. If not, cases of low population immunity may still be identified, however, the antigenic similarity of the vaccine, the test and the field virus should be considered in detail.

Starting vaccination with two vaccine doses, no less than one month apart, would dramatically increase population immunity, particularly in young animals. This is now conducted in certain provinces in Turkey and higher potency vaccines ($\geq 6PD_{50}$) are now routinely used throughout the country. Evaluation of this new strategy is required.

Conflict of interest statement

Dr A.N. Bulut is employed by the Şap institute, which manufactures the vaccine under-evaluation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.12.010.

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