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Immune response to an alum precipitated haemorrhagic septicaemia vaccine in buffaloes at a semiorganized farm of Madhya Pradesh in India

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Abstract: Haemorrhagic septicaemia (HS) is one of the economically most important bacterial diseases of large ruminants. In the present investigation, a total of 156 buffaloes (Murrah, Jafarabadi, and Bhadawari breeds) at a semiorganized farm after administration of HS alum precipitated killed vaccine were screened serologically for the presence of anti-HS antibodies by single dilution indirect enzyme linked immunosorbent assay (iELISA). Out of 156 buffaloes tested for presence of anti-HS antibodies, a greater proportion of buffalo (67.94%) had protective level of anti-HS antibodies even after 90th day post vaccination.

Key words: Haemorrhagic septicaemia, immune response, vaccine

1. Introduction

Haemorrhagic septicaemia (HS) is one of the economically most important bacterial diseases mainly of cattle and buffaloes. The disease is caused by a gram-negative coccobacillus *Pasteurella multocida* subsp. *multocida* belonging to the family *Pasteurellaceae* [1,2]. In India and Africa, serotypes B:2 and E:2, respectively are responsible for causing HS in large ruminants [3], although serotypes A:1 and A:3 have also been linked. HS affected buffalo exhibit respiratory sounds, profuse salivation, dyspnoea, mucous nasal discharge, high temperature, reduced appetite, restlessness, mandibular and neck region oedema, and redness [4]. According to 19 th livestock census (2012), the total bovine population was 299.9 million in India (http://dahd.nic.in/sites/default/filess/Livestock%20%205_0.pdf). Out of this, a significant percentage (approximately 36%, 108.7 million) consisted of buffalo, which makes India rank first in the buffalo population in the world. Nearly half of this buffalo population (51.05 million) consists of milch buffalo contributing around 50% of the total milk production. India is the largest producer of buffalo milk and contributes 68% of the total world buffalo milk produced [5]. As per the Department of Animal Husbandry, Government of Madhya Pradesh, the state ranks fourth in the country for milk production (10.78 million tonnes during the year 2014–2015) with 383 g milk availability per capita per day, higher than the national average of 313 g (http://www.mpdah.gov.in/upload/10_year_achievement_with_graph.pdf). The total national gross domestic product (GDP) contribution of livestock sector was 5.26% while livestock in Madhya Pradesh contributes a quite higher 8%–10% to the GDP of the state. In 2016, India received US $ 3810 million export value of buffalo meat. Estimated economic losses due to HS in India are to the tune of US $ 750 million [6]. In India, HS vaccination is routinely practiced in large ruminants for prevention and control of the disease in endemic areas and contain the losses. According to the Department of Animal Husbandry Dairying and Fisheries, Government of India, there was a significant reduction (more than 50%) in the number of HS outbreaks from 698 (2011–2014) to 300 (2014–2017) (http://dadf.gov.in/sites/default/filess/New%20initiatives%20for%20doubling%20farmers.pdf). Microtiter agglutination test (MAT), indirect hemagglutination assay (IHA), and enzyme linked immunosorbent assays (ELISA) are usually employed to detect serum antibody levels in immunized animals [7]. The ELISA detects immune response to soluble antigens and generally used to detect IgG antibodies. The present study reports antibody response against alum precipitated HS vaccine in buffaloes reared at a semiorganized farm of Madhya Pradesh.

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2. Materials and methods

2.1. Geographical location

Madhya Pradesh State Livestock and Poultry Development Corporation has a semi-organized Animal Breeding Farm at Kiratpur–461111 (22.5395° N, 77.7645° E), Itarsi, District Hoshangabad, Madhya Pradesh. At the time of this study, the farm had 250 buffaloes and 6 cattle. The place has a subtropical climate; a hot dry summer (April–June) followed by monsoon rains (July–September), and a cool and relatively dry winter [8].

2.2. Vaccine

HS alum precipitated killed vaccine produced by Institute of Animal Health and Veterinary Biologicals (IAH&VB), Rasalpura, Mhow-453446, Indore, Madhya Pradesh was used in the present study.

2.3. Animals

A total of 156 adult buffalo of Murrah, Jafarabadi, and Bhadawari breeds (>3 years of age) being reared at the Animal Breeding Farm, Kiratpur were used in the present study. The buffaloes were administered with HS alum precipitated killed vaccine @5mL by following the subcutaneous route in the month of June in 2015. Animals received the first vaccine after attaining the age of 6 months and thereafter a booster dose every year.

2.4. Collection of blood samples, separation of serum, and transportation and processing of samples

Blood (8–10 ml) was collected aseptically from the jugular vein of each buffalo only once on the 90th day post vaccination, allowed to clot and serum was collected in the sterile cryovials. Containers were allowed to clot under the shade for half an hour duration. The serum samples were transported under cold chain conditions, initially at the Animal Breeding Farm, Kiratpur; then to Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Mhow-453446, Madhya Pradesh. Thereafter these samples were carried to the Department of Veterinary Microbiology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar – 125004, Haryana under cold chain conditions and were stored at deep freezing conditions (–20 °C) till used.

2.5. Single dilution indirect ELISA

The single dilution indirect ELISA (iELISA) for detection of serum antibodies against *Pasteurella multocida* (causing haemorrhagic septicaemia) developed in the Department of Veterinary Microbiology, COVS, LUVAS, Hisar was employed in the present investigation and briefly described.

2.5.1. Coating of microplates with antigen

The ELISA microplates were coated with sonicated antigens of *Pasteurella multocida* (approx. 1 ng/ well). The antigen coated plates were incubated at 37 °C for 1 h and transferred to 4 °C. Next day, the ELISA plates were washed by flooding wells with wash buffer and decanting for 3 cycles and were finally tapped to dry. Meanwhile, the buffalo sera samples were kept at room temperature for thawing.

2.5.2. Testing of serum samples by iELISA

For dilution of sera samples, 50 µL of the diluent buffer was added to well A1 and 45 µL to all the remaining wells of the ELISA plate. 5 µL of each negative and positive serum controls were added to the B1 to F1 and G1 to H1 wells, respectively. Now, 5 µL of each test serum sample (1-88) was added to the respective wells (A2 to H12) of the ELISA plate (final volume of 1:10 in 50 µL volume). The plates were incubated at 4 °C for overnight incubation. Next day, the ELISA plates were washed as described earlier and 50 µL of the 1:1000 tracing antibody (monoclonal antibody cross reacting equally with buffalo and cattle IgG) was added in all the wells of the ELISA plates and incubated at 37 °C for 1 h. After incubation, the plates were washed and dried as described earlier, followed by addition of 50 µL of the 1:10,000 goat anti-mouse IgG horseradish peroxidase (HRPO, Sigma Aldrich) conjugate and were incubated at 37 °C for 1 h. After washing and drying, 50 µL of the 1:100 diluted stock TMB substrate solution was added in each of the wells and plates kept in the incubator at 37 °C for 5 min for development of the blue colour in positive cases. The colour development reaction was stopped by adding 50 µL of stopping solution (1 M H_2SO_4) to each well. Optical density (OD) of each well was measured by ELISA Reader (Tecan, Austria) at 450 nm and the antibody titres (log_{10}) were calculated.

2.6. Interpretation

The results were interpreted as follows: the serum samples with antibody titers <1.50 log_{10} were considered as 'not protected'; antibody titers between 1.5 log_{10} and 1.80 log_{10} as 'partially protected', and those with antibody titer >1.8 log_{10} as 'protected'.

3. Results

Serum antibody titres ranging from 1.4803 log_{10} to 2.2351 log_{10} were observed by iELISA for the presence of antibodies against HS [Table 1, Table 2, Figure 1]. Of the total 156 buffaloes, 4, 52, and 100 buffaloes demonstrated serum antibody titres <1.5 log_{10} (1.4803 log_{10} to 1.4892 log_{10}), 1.5 log_{10} to 1.8 log_{10} (1.5296 log_{10} to 1.7922 log_{10}) and >1.8 log_{10} (1.7997 log_{10} to 2.2351 log_{10}), and were categorized as 'not protected', 'partially protected' and 'protected' against HS, respectively [Figure 2]. Among the 100 buffaloes categorized as 'protected' a larger proportion of animals had serum antibody titres in the range 1.8 log_{10} to 1.9 log_{10}.

Table 1. Antihaemorrhagic septicaemia antibody titres in serum from buffaloes vaccinated with alum precipitated haemorrhagic septicaemia killed vaccine by single dilution indirect enzyme linked immunosorbent assay and not-protected/partially protected.

Buffalo	Antibody titre	Buffalo	Antibody titre	Buffalo	Antibody titre
179*	1.4803	283	1.6371	56	1.6987
1516*	1.4867	Uk20	1.6445	119	1.7017
9*	1.4879	98	1.6447	189	1.7308
164*	1.4892	135	1.6452	8	1.7405
1512	1.5296	176	1.6488	41	1.7410
148	1.5375	190	1.6566	Uk10	1.7412
183	1.5532	174	1.6604	1010	1.7488
285	1.5547	289	1.6616	223	1.7584
2516	1.5717	286	1.6637	186	1.7718
193	1.5719	1581	1.6644	54	1.7766
91	1.5726	147	1.6664	192	1.7814
115	1.5798	168	1.6716	292	1.7814
167	1.5807	267	1.6733	1513	1.7821
5	1.5886	85	1.6743	57	1.7823
2519	1.6121	44	1.6787	127	1.7885
2504	1.6158	4	1.6916	132	1.7886
18	1.6248	172	1.6917	2507	1.7911
2505	1.6267	242	1.6955	225	1.7922
2517	1.6317	6	1.6966		

*Not protected, rest-partially protected

4. Discussion

Vaccination of animals not only protects susceptible population from the infection but also by means of herd immunity that confers indirect protection to the unvaccinated population. Increased level of humoral immunity or antibody response to the immunization prevents circulation of infectious agent in susceptible populations [9,10]. Vaccination is the key to prevent and control outbreaks of HS in susceptible livestock population in endemic areas. Buffaloes are more susceptible than cattle to HS caused by *P. multocida*, and hence it is of utmost importance to vaccinate these animals specifically those being reared at semiorganized or organized farms in large numbers. It is suggested to implement control strategies in buffalo dominated areas with a higher priority [11]. Buffalo from Madhya Pradesh contributed 4309.19 thousand tonnes of milk production and 15.1 thousand tonnes of meat production [12]. Considering the economic contribution from buffalo to Madhya Pradesh state economy to reduce losses due to HS various kinds of single (alum precipitated/aluminium hydroxide gel/mineral oil adjuvanted) and combined (combined foot-and-mouth disease-haemorrhagic septicaemia-Blackquarter) HS vaccines are continuously being developed and tested for immunization of livestock against these diseases in the endemic areas world-wide including India [13].

Bacterins from plain broth, or alum precipitated and aluminium hydroxide gel vaccines, oil adjuvant vaccine, live vaccines and subunit vaccines are used in veterinary practices for prevention and control of various diseases in animals. Due to its availability in the state, cost effectiveness, and ease in injection [14,15,16], HS alum precipitated vaccine is the most commonly used vaccine for immunization of susceptible livestock population for prevention and control of HS caused by *P. multocida*. There was an increased awareness for vaccination as an overall including against HS in Madhya Pradesh. Approximately 207.08 lakh vaccine doses were administered in 2014–2015 compared to 70.79 lakh vaccine doses in 2005 owing to better animal husbandry services. Knowledge of the proportion of immune to nonimmune animals and the levels of antibodies in the herd will be helpful for ascertaining the severity of the outbreak and initiation of possible prophylactic and therapeutic measures for

Table 2. Antihaemorrhagic septicaemia antibody titres in serum from protected buffaloes vaccinated with alum precipitated haemorrhagic septicaemia killed vaccine by single dilution indirect enzyme linked immunosorbent assay.

Buffalo	Antibody titre	Buffalo	Antibody titre	Buffalo	Antibody titre	Buffalo	Antibody titre
163	1.7997	1582	1.8274	224	1.8478	145	1.8921
138	1.8006	128	1.8288	38	1.8482	2502	1.8939
2503	1.8014	1559	1.8296	49	1.8484	180	1.8942
62	1.8028	1582	1.8325	123	1.8491	143	1.8943
197	1.8032	39	1.8327	10	1.8493	117	1.8974
17	1.8043	30	1.8338	1518	1.8542	290	1.8978
117	1.8054	181	1.8342	2508	1.8595	36	1.9124
121	1.8089	2506	1.8345	268	1.8600	1057	1.9185
195	1.8105	50	1.8346	1562	1.8611	134	1.9188
2511	1.8109	130	1.8347	165	1.8632	300	1.9227
21	1.8116	22	1.8352	236	1.8676	2515	1.9272
177	1.8116	169	1.8357	118	1.8703	87	1.9294
3	1.8119	144	1.8359	188	1.8732	102	1.9522
146	1.8127	1511	1.8361	178	1.8773	254	1.9688
37	1.8148	99	1.8367	116	1.8781	140	1.9739
149	1.8151	162	1.8367	45	1.8801	142	1.9774
88	1.8163	2512	1.8371	141	1.8809	269	1.9897
2501	1.8182	1315	1.8372	173	1.8811	139	1.9946
185	1.8189	288	1.8383	171	1.8813	1520	1.9967
2514	1.8208	229	1.8391	136	1.8825	175	2.0037
86	1.8212	1518	1.8396	194	1.8838	124	2.0056
184	1.8215	2513	1.8404	191	1.8866	161	2.0118
48	1.8218	259	1.8422	150	1.8877	100	2.0124
176	1.8232	133	1.8439	226	1.8896	89	2.0202
1519	1.8238	132	1.8474	287	1.8897	51	2.2351

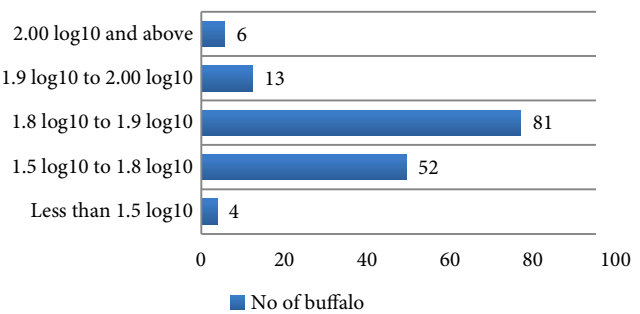

Figure 1. Number of buffalo showing antihaemorrhagic septicaemia antibody titres in serum vaccinated with alum precipitated killed haemorrhagic septicaemia vaccine.

future disease outbreak(s), if any. It is necessary to monitor vaccinated animals to ascertain protective serological response in individual animal as well as at herd level. Although for measurement of immunity in vaccinated animals, passive mouse protection test, IHA and ELISAs are available. ELISAs have been considered to be the most suitable assay for screening large numbers of serum samples to detect humoral response against vaccination [17,18].

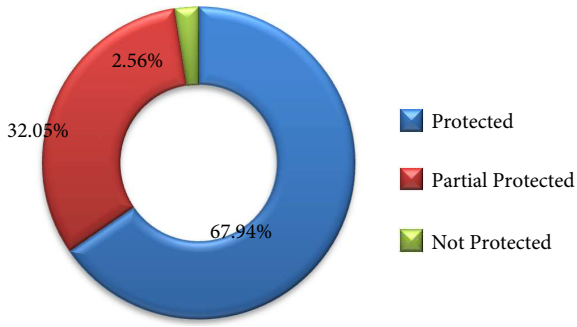

Figure 2. Percent of buffalo showing different protection levels of antihaemorrhagic septicaemia antibody titres in serum vaccinated with alum precipitated killed haemorrhagic septicaemia vaccine.

Hence, a serological test, iELISA developed indigenously was employed for screening the buffalo population immunized with alum precipitated HS killed vaccine to evaluate anti-HS antibody titers and categorized into one of the criteria: 'not protected', 'partially protected', and 'protected' against HS. In the present investigation, 67.94%, 32.05%, and 2.56% buffalo were grouped in one of the 3 groups i.e. 'protected', 'partially protected', and 'not protected', respectively on 90 days post primary vaccination against HS.

Various reports indicated maintenance of varied duration of reliable immunity from 3 to 9 months in the vaccinated animals [18]. In the present study, quite a large proportion of buffaloes (106) were categorized as 'protected' but a significant proportion (52) remained 'partially protected', and few (4) 'not protected'. Booster vaccination after priming the animals had shown to confer higher duration of protection [19]. Higher antibody titers up to 14 months postvaccination have been reported in animals vaccinated with HS subunit vaccine though such vaccines are not available commercially in India. Aerosol vaccines (aerosol intranasal spray of live vaccines) were shown to protect buffalo calves for 7 months post vaccination against HS. The duration of protection against challenge increased up to 12 months when the same animals were given booster dose one month after primary vaccination [20]. In India, herbal adjuvant based vaccinated animals showed high levels of antibody titres 180 days post vaccination as compared to traditional alum precipitated vaccine (reduced antibody titres after 150 days postvaccination) against HS [21]. In one of the studies, researchers opined for annual back passage for the vaccine seed culture to prepare vaccines with improved potency [22].

Since occurrence of HS is higher in buffalo specifically in young calves, this species needs to be immunized regularly in a systemic manner [23]. Though the buffalo in the present study were regularly vaccinated for protection against HS and proper feeding practices were employed, they were rarely monitored for trace minerals and vitamin deficiencies, if any. Positive effect of vitamin E and selenium supplementation on antibody titres to HS vaccine was studied [24]. Hence, in our study the buffalo having low levels of antibody titres might be attributed to nutritional deficiencies. Immunosuppressive effect to haemorrhagic septicaemia vaccination in *T. evansi*-infected buffalo-calves were also found [25]. In future, such detailed studies on buffalo population of the state can be planned. Therefore, even after vaccination the animals must be closely monitored by physical examination and serological testing and standard livestock managemental practices including deworming must be implemented [26]. Combined HS and FMD vaccines produced better antibody titer which lasted for longer duration [27]. Large ruminants in Madhya Pradesh are also affected by FMD. Hence, it will be worthy to administer combined vaccines for protection against HS and FMD to buffalo of Madhya Pradesh in India. Further, use of combined vaccines (HS and FMD/HS, FMD and BQ) are better suited to minimize efforts required in vaccinating animals.

Based on the findings in the present study, it can be concluded that higher (67.94%) herd immunity against HS was detected in buffalo from the semiorganized farm. The present study will help to understand the formulation of better vaccination strategies for prevention and control of HS in buffalo of Madhya Pradesh in India.

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References

1. The Center for Food Security and Public Health. Hemorrhagic septicemia. Ames, IA, USA: Iowa State University, College of Veterinary Medicine; 2009.
2. Karunasree P. A brief study on hemorrhagic septicemia. Research & Reviews: Journal of Veterinary Sciences 2016; 2 (2): 30-37.
3. Charan S, Batra SK. Prerequisites for the control of infectious diseases with special reference to hemorrhagic septicemia in bovine. In: Sharma R, Kakker NK (editors.) Course Compendium: Control of Infectious Diseases in Animals With Particular Emphasis on FMD Control Programme. Hisar, India: ICAR Center for Advanced Faculty Training, Department of Veterinary Microbiology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences; 2012. pp. 80-86.
4. Khan A, Saleemi MK, Khan MZ, Gul ST, Irfan M et al. Hemorrhagic Septicemia in buffalo (*Bubalus bubalis*) calves under subtropical conditions in Pakistan. Pakistan Journal of Zoology 2011; 43 (2): 295-302.
5. Islam MR, Sharma B, Dem P, Pattnaik B, Jha VC et al. Economic impact of TADs in India. In: Siddiky MNA (editor). Economic Impact of Transboundary Animal Diseases in SAARC Countries. Dhaka, Bangladesh: SAARC Agriculture Centre; 2013. pp. 37-38.
6. Pal R, Kumar S, Bardhan D, Goyal J, Anandasekaran G et al. Quantifying morbidity and mortality losses in bovine due to Haemorrhagic Septicemia (HS) in Haryana. The Pharma Innovation Journal 2018; 7 (7): 802-806.
7. Qureshi S, Saxena HM. Estimation of titers of antibody against *Pasteurella multocida* in cattle vaccinated with haemorrhagic septicemia alum precipitated vaccine. Veterinary World 2014; 7 (4): 224-228.
8. Madhya Pradesh State. Agricultural Portal. New Delhi, India: Government of India; 2012.
9. Kim TH, Johnstone J, Loeb M. Vaccine herd effect. Scandinavian Journal of Infectious Diseases 2011; 43 (9): 683-689.
10. Fine P, Eames, K, Heymann DL. Herd immunity: a rough guide. *Clinical Infectious Diseases* 2011; 52 (7): 911-916.
11. Shivachandra S, Viswas K, Kumar A. A review of hemorrhagic septicemia in cattle and buffalo. Animal Health Review 2011; 12 (1): 67-82.
12. Haidari H. Dynamics of livestock sector in Madhya Pradesh-an economic analysis. MSc, Jawaharlal Nehru Krishi Vishwa Vidyalaya, India, 2016.
13. Benkirane A, De Alwis MCL. Haemorrhagic septicemia, its significance, prevention and control in Asia. Veterinary Medicine-Czech 2002; 47 (8): 234-240.
14. Lubroth J, Rweyemamu MM, Viljoen G, Diallo A, Dungu B, et al. Veterinary vaccines and their use in developing countries. Revue Scientifique Et Technique Office International Des Epizooties 2007; 26 (1): 179-201.
15. Maslog FS. Immunity provided by haemorrhagic septicemia-subunit vaccine in ruminants. Developments in Biological Standardization 1998; 9 : 301-307.
16. Verma R, Jaiswal TN. Haemorrhagic septicaemia vaccines. Vaccine 1998; 16 : 1184-1192.
17. Kharb S. Development of ELISA techniques for haemorrhagic septicemia. International Journal of Bioassays 2015; 4 (11): 4574-4577.
18. De Alwis MCL. Haemorrhagic Septicaemia Monograph No 57. Canberra, Australia: Australian Centre for International Agricultural Research; 1999.
19. Sarwar N, Mohammad K, Rabbani M, Rana MY, Sarwar M et al. Factors affecting potency of hemorrhagic septicemia vaccine. International Journal of Agriculture & Biology 2015; 17: 387-390.
20. Saleem L, Munir R, Ferrari G, Afzal M, Chaudhry R. Efficiency and cross- protectivity of live intranasal aerosol hemorrhagic septicemia vaccine in buffalo calves. International Journal of Current Microbiology & Applied Sciences 2014; 3 (11): 300-307.
21. Tanwar H, Yadav AP, Bhushan B, Shweta J, Singh SB et al. Immunity against *Pasteurella multocida* in animals vaccinated with inactivated *Pasteurella multocida* and herbal adjuvant 'DIP-HIP'. Journal of Vaccines & Immunology 2016; 2 (1): 10-1.
22. Gowrakkal M, Chandrashekar M, Bhajantri S, Satav J, Chandakala GC et al. Evaluation of immuno efficiency of hemorrhagic septicaemia vaccine strain (vaccine seed). Asian Pacific Journal of Tropical Biomedicine 2014; 4 (1): S263-S267.
23. Khan A, Saddique U, Ahmad R, Khan H, Mohammad Y et al. Sero-surveillance of hemorrhagic septicemia in cattle and buffaloes in district Malakand, NFWP. Journal of Agricultural & Biological Science 2006; 1 (4): 11-14.
24. Prince K, Khan MS, Ijaz M, Anjum AA, Prince A et al. Effect of vitamin E and selenium supplementation on antibody titer against to hemorrhagic septicemia vaccine in buffalo calves. International Journal of Veterinary Science 2017; 6: 26-30.
25. Singla LD, Juyal PD, Sharma NS. Immune responses to haemorrhagic septicemia (HS) vaccination in *Trypanosoma evansi* infected buffalo-calves. Tropical Animal Health & Production 2010; 42 (4): 589-595.
26. Elshemey TM, Abd-Elrahman AH. Hemorrhagic septicemia outbreak as a consequence to SAT2 FMD infection in buffalo and cattle in Alexandria Province, Egypt. Life Science Journal 2013; 10 (2): 816-822.
27. Altaf I, Siddique M, Muhammad K, Irshad M, Khan MZ et al. Antibody response of rabbits to combined hemorrhagic septicemia and foot-and-mouth disease virus vaccine. The Journal of Animal & Plant Sciences 2012; 22 (2): 501-504.