academicJournals

Vol. 12(6), pp. 141-146, 14 February, 2018
DOI: 10.5897/AJMR2017.8507
Article Number: 307D4CD56046
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evaluation of safety and immunogenicity of combined blackleg and hemorrhagic septicemia vaccine

Hunderra Sori*, Shiferaw Jemberie, Martha Yami, Jalata Shuka, Birhanu Abebe and Kenaw Birhanu

National Veterinary Institute, P. O. Box 19, Bishoftu, Ethiopia.

Received 2 March, 2017; Accepted 28 June, 2017

To evaluate the safety and immunogenicity of combined hemorrhagic septicemia (HS) and blackleg (BL) vaccine, combined vaccine was produced in two different formulations (group one, G1 and group two, G2) and compared with safety and immunogenicity of monovalent Hs and BL vaccines in different doses. G1 combined vaccine was vaccinated in 4 and 2 ml in to five calves each while G2 combined vaccine, the monovalent HS and blackleg vaccines were vaccinated in 2 and 1 ml amount into to five calves each leaving ten non-vaccinated calves managed similarly as vaccinated ones. The safety test was made by giving single and double doses of experimental vaccines and double doses of monovalent vaccines in each case using two calves per dose group. The immunogenicity of BL vaccine component in the combined vaccine was evaluated by using 10 guinea pigs per group for six different doses of combined and monovalent blackleg vaccine, leaving 10 non-vaccinated controls. The study indicated that the protection against virulent challenges for animals vaccinated with G1 combined vaccine at 2 ml, G2 vaccine at 1 ml, monovalent HS and BL vaccine vaccinated at 1 ml doses was by far less than 90%. On the other hand, protection against experimental challenge for G1 vaccine vaccinated in 4 ml amount was 100% against both HS and blackleg virulent challenges while the protection against experimental challenge for G2 vaccine vaccinated in 2 ml amount was 66.67% against the HS virulent challenge and 90% against BL virulent challenge. So G1 combined vaccine vaccinated in 4 ml was found to be the best candidate vaccine according to this experiment which needs to be confirmed at field test before use for mass vaccination.

Key words: Combined, hemorrhagic septicemia, blackleg, safety, immunogenicity.

INTRODUCTION

Veterinary vaccines have been known to be the most cost-effective tools for the prevention and control of infectious disease. Their uses have an enormous impact on disease both in eradication of the disease totally, as was seen in Rinder pest, and reducing the incident of disease occurrence, both meant to benefit the animal owners and the animal itself specifically and further the whole community and the country in general. There are multiples of diseases for which vaccines have been developed and vaccination is used to prevent and

*Corresponding author: E-mail: ayanahunderrasori@yahoo.com.

control both the incidence and impact of those diseases. However in developing countries like Ethiopia the vaccination coverage is not comparable with necessity and disease prevalence (personal observation). Among factors that reduce vaccination coverage, the budget (logistics) issue is mentioned as the primary one limiting the delivery of vaccines as a control means (to contain the outbreak) only (Stevenson, 2009).

Even though the government has to plan and implement mandatory vaccinations for animal disease prevention, other strategies which support the reduction of vaccination costs and facilitate the vaccination schedule are also necessary. One important approach is the use of combined vaccines (Stämpfli, 2016; Johns and Hutter, 2010; Dodd, 2003; Edwards, 1994) whose uses have the enormous advantages as it provides the ability to administer two or more antigens in a single shot, thus decreasing the discomfort for vaccine recipients and owners by reducing number of injections, reducing the cost of the vaccine delivery process as it reduces number of contacts, increasing vaccination coverage for the vaccines concerned as it could minimize chances of dropouts and also saves budget to cover other sites, and aiding to make the vaccination schedule safe that in turn minimizes the frequency of animal gathering and thereby decrease chance of disease transmission (Thrusfield, 1995).

A combined vaccine has been known to be prepared by mixing two or more live or inactivated or purified antigens at one of the end production processes or by mixing two vaccines at the time of application of the vaccines to prevent multiple diseases or to prevent one disease caused by different strains or serotypes of the same organism (Pastoret et al., 1997).

Epidemiologically it has been known that hemorrhagic septicemia occurs following stressing conditions especially at the beginning of rainy seasons when animals are at poor body condition and throughout the rainy season if animals are at poor body condition as this season is conducive for the transmission of pasteurella organisms (Radostits et al., 2007). On the other hand even if blackleg has been known to occur throughout the year, it is worse in spring and in fall. This indicates that disease prevention activities for both diseases. Blackleg and hemorrhagic septicemia could be synchronized and vaccination against both diseases could be made together. In Ethiopia despite the presence of constant threat both by blackleg and hemorrhagic septicemia, the vaccine comprising both antigens in single preparation is absent even if important. A report by Jabari et al. (2008) indicated that there is evidence of production of potent combined blackleg and hemorrhagic septicemia vaccine in Iran. Similarly according to different researchers (Srinivasan et at al., 2012; Ghanem and Ghanem, 1987), the immunity conferred by combined vaccine of blackleg and hemorrhagic septicemia was similar to that obtained by each vaccine alone. The main objective of this study is therefore to evaluate the safety and immunogenicity of combined hemorrhagic septicemia and blackleg vaccine produced under experimental batch.

MATERIALS AND METHODS

Description of the study area

This experimental study was done in National Veterinary Institute (NVI) in Bishoftu town which is situated 47 km south east of the capital city, Addis Ababa. It was found at 9°N latitude and 4°E longitudes at an altitude of 1850 m above sea level in central highlands of Ethiopia (NMSA, 1999). National veterinary Institute is the sole veterinary vaccine producing laboratory in Ethiopia and currently produces over 260 million doses of veterinary vaccines each year.

Laboratory animals management and antibody screening

52 male calves obtained from market and 70 male guinea pigs which were bought from Ethiopian Public health institute (EPHI) were used for this study. The animals were given a quarantine time of two weeks before the start of the actual experiment. During the quarantine period and thereafter during the experiment the animals were kept on management practice recommended for these animals (housing, feeding and watering). Calves were screened for *P. multocida* type B antibodies ahead of the experiment.

Experimental design

Laboratory animals grouping

Guinea pigs: 70 guinea pigs of 300 to 400 g size were used to test the immunogenicity of blackleg component.

CALVES: 40 calves aged between 6 month and 1.5 year was used for immunogenicity test while 12 calves of similar age group were used for safety test. Sera samples were taken from these calves and screened for presence of *P. multocida* type B2 antibody by indirect haemagglutination test according to OIE (2012). Those which were seronegatives were used.

Combined blackleg and hemorrhagic septicemia vaccine production

Clostridium chauvoei antigen preparation

18 L *C. chauvoei* inoculums media was inoculated with known *C. chauvoei* seed (local isolate) and incubated overnight after which sample was aseptically taken in class II level biological safety cabinet and checked for purity by Gram's stain. Then this inoculum was added to sterile 400 L liver and meat broth in 500 L size capacity fermenter. 50% glucose was also added to act as a carbon source in 9 L. The whole mix is incubated at 37°C for 24 h and when it showed full growth, sample was taken and purity checked and inactivated with 37% formalin at rate of 0.7% of culture volume. Then inactivation test and purity test were made both in guinea pigs and in laboratory media according to standard operating procedure for production of blackleg vaccine (Birhanu, 2015a, b; Tsetarge,

2015a, b; Misra, 1991).

Hemorrhagic septicemia antigen preparation

18 L *Pasteurella multocida* inoculum media was inoculated with known *P. multocida* type B:2 (local isolate used by NVI), and incubated overnight after which sample was aseptically taken in class II level biological safety cabinet and checked for purity by Gram's staining. Then this inoculum was added to sterile 400 L liver and meat broth in 500 L size capacity fermenter. Glucose (2.5%) was also added to act as a Carbone source in 9 L amount and 3 L sterile horse serum was added to aid the growth. The whole mix was incubated at 37°C for 18 to 20 h and when it showed full growth, sample was taken and checked for purity and inactivated with 37% formalin in 0.5% amount. Then inactivation test and purity test were made both in rabbits and in laboratory media (Birhanu, 2015a, b; Tsetarge, 2015a, b; OIE Manual, 2012).

Preparation of combined vaccine

Group one (G1) combined hemorrhagic septicemia and blackleg vaccine was prepared by mixing the separately mixed 9 L clostridium chauvoei antigen with 1 L 10% aluminium potassium sulphate adujuvant with separately mixed 5 L *P. multocida* type B:2 antigen with 4 L saline and 1 L 10% aluminum potassium sulphate adjuvant: that is in equal proportion which is dispensed in 100 ml polypropylene vials.

Group two (G2) combined vaccine was prepared by mixing nine liter clostridium chauvoei, nine liter *P. multocida* type B:2 antigen and 2 L of 10% aluminum potassium sulphate and dispensing the mix in 100 ml polypropylene vials.

The remaining of the bulk in separate fermenters containing *C. chauvoei* and *P. multocida* type B:2 was used to prepare monovalent blackleg and hemorrhagic septicemia vaccine respectively part of which is utilized as positive control during safety and immunogenicity test of the combined vaccine following the usual procedure of the National veterinary institute, Ethiopia (Birhanu, 2015a, b; Tsetarge, 2015a, b).

Purity and inactivation tests

The combined vaccines (G1 and G2) and monovalent vaccines were tested for freedom from any contaminant on VF media, 10% horse serum supplemented tryptose soy broth and agar incubated both aerobically and anaerobically, and saboraud agar (Birhanu, 2015a, b; Tsetarge, 2015a, b; OIE Manual, 2012; Misra, 1991).

Safety test

The combined vaccines (G1 and G2) were tested as G1 in 4 and 8 ml doses per animal; G2 in 2 and 4 ml doses per animal and the monovalent hemorrhagic septicemia and blackleg vaccine was given in 4 ml for safety test and test animals were monitored for three weeks (Birhanu, 2015a, b; Tsetarge, 2015a, b; OIE Manual, 2012 and Misra, 1991).

Immunogenicity test

All vaccine groups were subjected to sterility and safety testing before using them for immunogenicity test according to British Veterinary Pharmacopoeia (2010). Immunogenicity test was done separately for hemorrhagic septicemia and blackleg vaccine components:

Immunogenicity of hemorrhagic septicemia component: 40 calves were used for the immunogenicity test of the haemorrhagic septicemia component of the combined vaccine (10 animals each for G1 and G2), 10 animals for monovalent haemorrhagic septicemia vaccine as positive control and 10 calves as negative control (non-vaccinated). The G1 combined vaccine was vaccinated in 2 and 4 ml per dose while the G2 combined vaccine was vaccinated in 1 and 2 ml while the monovalent Hemorrhagic septicemia vaccine was vaccinated in 2 and 1 ml per animal. These animals were followed for nine months and challenged twice (between 5-6th month and at 9th month post initial vaccination) subcutaneously with 1 ml culture containing 5×10^7 CFU of virulent *P. multocida* type B: Three animals were challenged per group each time according to (Indian Pharmacopeia).

Post challenge, the calves were observed for evidence of clinical symptoms of hemorrhagic septicemia and death until 7 to 10 days. The animals' rectal temperature was also recorded twice in the morning and in the afternoon. The body temperature was termed febrile if > 39.5°C (Radostits et al., 2007).

Immunogenicity of Blackleg component of the combined vaccine: Seventy (n=70) 300 to 400 g weighing guinea pigs were used to test the immunogenicity of the blackleg component of the combined vaccine and monovalent blackleg vaccine (G1 in 2 and 4 ml; G2 in 1 and 2 ml; monovalent blackleg in 1 and 2 ml) each group comprising 10 guinea pigs including the non-vaccinated control. According to Indian pharmacopeia (http:ipc.nic.in/super/users/writecomm1main.asp), booster dose was given after 28th days of initial vaccination subcutaneously and challenged with 2 ml of pure clostridium chauvoei after 14th day of giving booster dose.

Post challenge, the guinea pigs were observed for evidence of clinical symptoms of blackleg like swelling of muscel with crepitating sound and death until 7 to 10 days (Radostits et al., 2007).

Bacterial isolation from clinical cases: Recently dead or clinically sick calves were thoroughly gross examined and samples like spleen, heart, heart blood and liver were taken and the cause of death was identified whether it was the organism used for challenge, *P. multocida* type B2 via proper *Pasteurella multocida* isolation and identification procedure (Quinn et al., 2002). Similarly from recently dead or seriously sick guinea pigs, swab sample taken from muscel and liver was cultured and culture suspension was tested by PCR to confirm whether the cause of death was *C. chauvoei* or not (Viljoen et al., 2005),

Statistical analysis

The complete data concerning vaccine combination and associated quality control tests (inactivation, freedom from contaminant, safety, immunogenicity and potency tests) were carefully recorded in Ms Excell spreadsheet (Microsoft office 2007 and analyzed using descriptive statistics (SPSS version 20).

RESULTS

Vaccines purity and inactivation tests

No growth of any organism was detected after inoculation of the prepared vaccines in VF or meat and liver broth media, 10% horse serum supplemented tryptose soy

Table 1. Percentage protection of calves with virulent *P. multocida* type B2 challenge between fifth and six month post vaccination.

Animal group	No. challenged	Vaccination volume/dose (ml)	Challenge dose (CFU/ml)	Observation post challenge	Conclusion
G1	3	4	5×10^7	All survived	100% protection
G2	3	2	5×10^7	2/3 survival	66.7% protection
G4 (+ve control)	3	2	5×10^7	All survived	100% protection
negative control	3	0	5×10^7	All died	No protection

Only calves vaccinated with higher dose per group were challenged this time. This is because the performance of lower vaccine doses was found poor during challenge for the blackleg vaccine component challenge trial. So animals which were vaccinated with lower volume per group were removed from the test animals.

Table 2. Challenge test result of Guinea pigs vaccinated with different vaccine doses.

Features	G1		G2		G3 (positive control)		Control
	4 ml	2 ml	2 ml	1 ml	2 ml	1 ml	
Death	0	3 (30%)	1 (10%)	3 (30%)	0	1 (10%)	5 (50%)
Serious subcutaneous/abdominal edema leading to wounding	0	2 (20%)	0	0	0	2 (20%)	5 (50%)
If animals with s/c or abdominal edema were euthanized as per recommendation, mortality rate was	0	50%	10%	30%	0	30%	100%
Protection	100	50	90	70	100	70	0

Challenge test and result interpretation was made according to the pharmacopeia (http:ipc.nic.in/super/users/writecomm1main.asp). The seriously sick animals were euthanized to minimize suffering and considered as dead.

broth and agar, sabouraud agar. These results indicated that the vaccines were free from any bacterial and fungal contaminant and indicated also the vaccine to be adequately inactivated.

Vaccines safety test

Observation of all the animals vaccinated with the combined and monovalent vaccines indicated that the combined vaccines were safe except minor swelling observed at the injection site which completely subsides within 2 to 3 weeks. Except this minor local reaction, the vaccinated animals showed no abnormal reaction to the vaccines.

Immunogenicity of the trial vaccines

Immunogenicity of hemorrhagic septicemia component of the combined vaccine

Challenge test: The result of challenge test for the hemorrhagic septicemia component between 5th and 6th month post vaccination showed that the G1 combined vaccine given in 4 ml had 100% protection where out of three calves all (100%) were protected from the active challenge similar to the monovalent hemorrhagic septicemia vaccine while the G2 combined vaccine given in 2 ml per dose showed ⅔ (66.67%) protection only. Similar challenge made on the ninth month post-vaccination showed a 50% decline in immunogenicity for both combined and monovalent Hemorrhagic septicemia vaccine (Table 1). Calves vaccinated with G1 combined vaccine in 2 ml, G2 combined vaccine in 1ml and monovalent hemorrhagic septicemia vaccine in 1 ml were not challenged because these vaccine doses performed poorly in Blackleg component challenge test on guinea pigs.

Immunogenicity of the Blackleg component of the combined vaccine

The result of the immunogenicity test of the blackleg component of the combined vaccine showed that G1 combined vaccine had 100% protection in 4 ml per dose while the G2 combined vaccine had 90% protection in 2 ml amount. The monovalent Blackleg vaccine had also 100% protection to virulent *C. chauvoei* challenge. The other vaccination groups revealed a far less protection capability (Table 2).

Challenge tests for trial vaccines

The result for Blackleg component (Table 2) made us to

Table 3. Challenge test result summary for trial vaccine groups.

Test	Vaccination group				Controls
	G1 (4 ml) combined	G2 (2 ml) combined	Mono-valent blackleg vaccine batch BL32/12	Monovalent HS vaccine batch PB17/12	
Protection for blackleg component	100%	90%	100%	N/A	100% death
Protection for hemorrhagic septicemia component	100% b/n 5th and 6th month post vaccination	66.7% b/n 5th and 6th month post vaccination	N/A	100% b/n 5th and 6th month post vaccination	100% death
	50% b/n 9th and 10th months post vaccination	50% b/n 9th and 10th months post vaccination	N/A	50% b/n 9th and 10th months post vaccination	100% death

N/A is not applicable

reject other lower vaccine doses and made us to give attention to G1 (4 ml), G2 (2 ml), G3 (2 ml) and negative controls only. As indicated for calf challenge in Table 1 for hemorrhagic septicemia component and guinea pig challenge in Table 2 for blackleg component, the challenge test result showed that G1 (4 ml) and G2 (2 ml) combined vaccines were better candidate combined vaccines. However, G1 (4 ml) had better performance than G2 (2 ml). Table 3 summarizes the result of both challenge tests and depicted that G1 in 4 ml combined vaccine to be the better performing trial combined vaccine group.

Bacterial isolations

Samples taken from jugular vein (whole blood), tissues (heart, liver, spleen, kidney and lymph nodes) and thoracic fluid from seriously sick or dead calves and cultured using routine bacteriological techniques and revealed re-isolation of the challenge organism, *P. multocida* type B:2. Similarly samples taken from muscel and edematouse abdominal fluid of severily ill guinea pigs and cultured anaerobically and tested by use of PCR revealed the reisolation of *C. chovoei*, as the challenge organism for blackleg component of the combined vaccine. This showed the re-isolation of organisms from sick animals or recently dead in both challenge components.

DISCUSSION

Haemorrhagic septicemia is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (OIE, 2012). Blackleg is an acute, febrile, highly fatal disease of cattle and sheep caused by *C. chauvoei* and characterized by emphysematous swelling, commonly affecting heavy muscles (*Clostridial myositis*) (Stampfli, 2016). These diseases have been known to be effectively prevented by vaccination with respective vaccines (Mosier, 2016; Stampfli, 2016). However, vaccination of animals by combining these two vaccines enables prevention of both diseases with a vaccine delivered via a single shot that reduces stress on the animals, financial, time and energy costs of the animal owner, and reduces chance of disease spread that could have been contracted via multiple times gathering of animals to vaccinate for HS vaccine and to vaccinate for blackleg vaccine at different times (Stämpfli, 2016; Johns and Hutter, 2010; Dodd, 2003; Thrusfield, 1995; Edwards, 1994).

In this trial, the safety and immunogenicity of combined hemorrhagic septicemia and blackleg vaccines formulated in two forms were evaluated in comparison to the respective monovalent hemorrhagic septicemia and blackleg vaccines. The result showed that the G1 combined vaccine given in 4 ml per dose gave 100% protection in hemorrhagic septicemia and blackleg component, the G2 combined vaccine in 2 ml per dose gave 66.7% protection in hemorrhagic septicemia component while 90% protection in blackleg component. The positive controls gave 100% protection for both hemorrhagic septicemia and blackleg vaccines. Ghanem and Ghanem (1987) reported the immunity conferred by combined vaccine of blackleg and hemorrhagic septicemia to be similar to that obtained by each vaccine alone. Other similar study made by Ardehali et al. (1997) proved the efficacy and safety of combined blackleg and hemorrhagic septicemia vaccines in both cattle and buffaloes. Srinivasan et al. (2012) in their study focused on evaluation of serological response of combined hemorrhagic septicemia, blackleg, foot and mouth disease, and rabies vaccines, found similar serologic response generated by combined vaccine and their respective individual components.

The challenge study in hemorrhagic septicemia component further indicated that the protection provided

is 100% between 5th and six month challenge while these figure falls to 50% at challenge made between 9 and ten months post vaccination. Similarly, Hanna et al. (2014) reported higher log protection against combined clostridia and pasteurella vaccines at six months than 7 months in sheep.

When the result of this study is seen over-whole, the group one vaccine in 4 ml/dose showed the better performance than group 2 combined vaccines in 2 ml/dose, even though the protection showed by group 2; 2 ml combined vaccine is also acceptable according to Indian pharmacopeia monograph (http://ipc.nic.in/super/users/writecomm1main.asp?id=508&cuid=&EncHid). A literature source on different manufacturers reveals that the blackleg and hemorrhagic septicemia combined vaccine is given in 4 ml amount per dose in cattle and buffaloes by Indian immunological limited (Raksha vaccine). In their study on the relationship between the response of guinea pigs and sheep following vaccination and challenge with virulent *C. chauvoei*, Crichton et al. (1986) found guinea pig laboratory model to be a valid indicator of field performance for vaccines containing blackleg antigen.

Therefore, according to the data generated by this experiments, the institute can produce combined vaccine for blackleg and hemorrhagic septicemia vaccine which could be given in 4 ml/dose in cattle after performing field level safety and immunogenicity tests.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Ardehali M, Aarabi I, Moosawi M, Sotoodenia A, Pilechian R, Mahinpour (1997). Immunization of cattle and buffaloes with a combined blackleg and hemorrhagic septicemia vaccine. Indian Vet. J. 74:1009-1011.

Birhanu K (2015a). Blackleg vaccine production procedure, NVI, Bishoftu.

Birhanu K (2015 b).Bovine pasteurellosis vaccine production procedure, NVI, Bishoftu.

British Veterinary Pharmacopeia (2010). The pharmaceutical Press, London.

Crichton R, Harriss DA, McKay DJ (1986). Standards for *Clostridium chauvoei* vaccine--the relationship between the response of guinea pigs and sheep following vaccination and challenge with virulent C. chauvoei. Aust. Vet. J. 63(3):68-70.

Dodd D (2003). Benefits of combination vaccines: effective vaccination on a simplified schedule. Am. J. Manage. Care 9(1):6-12.

Edwards KD (1994). Combination vaccines: hopes and challenges. Pediatr. Infect. Dis. J. 13:345-347.

Ghanem F, Ghanem M (1987). Immune response of animals for c*ombined black quarte*r and haemorrhagic septicaemia vaccine. Thesis Ph.D, Faculty Veterinary Medicine, Cairo University.

Hanna TN, Galila ELM, Ebeid MH, Houmda FK, Elmeneisy AA, Gorgy SF (2014). The effectiveness of bivalent clostridial and pasteurella combined vaccines in the laboratory animals. Benha Vet. Med. J. 27(2):302-308.

Indian pharmacopeia commission: (http://ipc.nic.in/super/users/writecomm1main.asp?id=508&cuid=&EncHid=)

Jabbari AR, Muazzeni Jula GR, Pileh Chiyan Lngrudi R, Musavi Shushtari M (2008). Preparation and evaluation of Improved blackleg and hemorrhagic septicemia vaccine. Agricultural Scientific Information and Documentation Centre, Agricultural Research and Education Organization. http://agris.fao.org/agris-search/search.do?recordID=IR2009000408.

Johns TL, Hutter GE (2010). New combination vaccines: DTaP-IPV (Kinrix) and DTaP-IPV/Hib (Pentacel). Ann. Pharmacother. 44(3):515-523.

Misra RP (1991). Manual for the production of anthrax and blackleg vaccines. FAO Animal production and health paper, Rome, FAO.

Mosier DA (2016). Overview of Hemorrhagic Septicemia. In. Merk Veterinary Manual. 11th eds. Merck & Co., Inc., Kenilworth, NJ, USA.

OIE Manual (2012): Hemorrhagic septicemia. In. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

Pastoret PP, Blancou J, Vannier P, Verschueren C (1997). Veterinary Vaccinology. Elsevier, New York.

Quinn PJ, Markey BK, Carter ME, Donnelly WJ, Leonard FC (2002). Veterinary Microbiology and Microbial Disease, Blackwell Science. pp. 137-143.

Radostits OM, Gray CC, Hinchliff KW, Constable PD (2007). A textbook of the disease of cattle, sheep, pig and goats.10th edition, Saunders Ltd. ISBN-13:978-0702027772.

Srinivasan VA, Reddy GS, Rao KA, Kihm U (2012). Serological response of bovines to combined vaccine containing foot and mouth disease virus, rabies virus, Pasteurella multocida and Clostridium chauvoei antigens. Vet. Arch. 71 (1):37-45.

Stämpfli HR (2016). Black leg. In. Merk veterinary Manual. 11th eds. Merck & Co. Inc., Kenilworth, NJ, USA

Stevenson A (2009). Factors limiting vaccination rates. Clinical Advisor. http://www.clinicaladvisor.com/features/factors-influencing-immunization-rates/article/156897/.

Thrusfield M (1995). Veterinary Epidemiology, second Edition. Blackwell Science Limited.

Tsetarge T (2015 a). Blackleg vaccine quality control procedure, NVI, Bishoftu.

Tsetarge T (2015b). Bovine pasteurellsis vaccine quality control procedure laboratory, NVI, Bishoftu.

Viljoen GJ, Nel LH, Crowther JR (2005). Molecular diagnostic hand book. FAO/IEA Eds.