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Full Length Research Paper

Spectral and statistical analysis of blood samples of sheep vaccinated with anthrax spore vaccine

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The application of spectroscopy for the study of biomedical compounds has increased tremendously in recent years. Blood is the chief circulatory medium in human and in animal body which can be subjected to non-invasive technique for testing. Pre and post vaccinated blood samples of sheep vaccinated with anthrax spore vaccine was studied using spectrometer. The internal standards among the application peaks were calculated. There was a marked difference in the absorption levels of the pre and post vaccinated blood samples. The resultant variation was attributed to the chemical changes that happened in the animal blood due to the vaccination. Spectral study can emerge as an alternate and cost effective test for screening animals. In future this study can be extended and compared with other antibody tests like ELISA (Enzyme-Linked Immuno Sorbent Assay).

Key words: Spectroscopy, anthrax spore vaccine, antibody, ELISA (Enzyme-Linked Immuno Sorbent Assay).

INTRODUCTION

Anthrax, a disease of mammals including human, is caused by a spore-forming bacterium called *Bacillus anthracis*. Most forms of the disease are lethal, and it affects both human and animals. Like many other members of the genus *Bacillus*, *Bacillus anthracis* can form dormant spores that are able to survive in harsh conditions for extremely long periods of time, even decades or centuries. Such spores can be found on all continents, even Antarctica (Hudson, 2006). When spores are inhaled, ingested, or come into contact with a skin lesion on a host they may reactivate and multiply rapidly. Anthrax commonly infects wild and domesticated herbivorous mammals which ingest or inhale the spores while grazing. Ingestion is thought to be the most common route by which herbivores contract anthrax. Carnivores living in the same environment may become infected by consuming infected animals. Diseased animals can spread anthrax to human, either by direct contact (e.g. inoculation of infected blood to broken skin or consumption of flesh of the diseased animal. *Bacillus anthracis* bacteria spores are soil-borne and because of their long lifetime, they are still present globally and at animal burial sites of anthrax-killed animals for many decades; spores have been known to have reinfected animals over 70 years after burial sites of anthrax-infected animals were disturbed (Cherkasskiy, 1999). Anthrax is even rarer in dogs and cats, there had only ever been one documented case in dogs in the USA by 2001, although the disease affects livestock (Dragon, 1999). Anthrax typically does not cause disease in carnivores and scavengers, even when these animals consume anthrax-infected carcasses. Anthrax outbreaks do occur in some wild animal populations with some regularity (Scott Shane, 2001). The disease is more common in developing countries without widespread veterinary or human public health programs. Vaccination is the best and cheapest method to protect the body against animal diseases. Anthrax spore vaccine (ASV) is a glycerinated suspension of live spores of uncapsulated a virluent strain of *B. anthracis*. ASV can be used to protect all species of animals viz cattle, sheep, goat and elephant.

Krishna et al. (2007) studied the evaluation of immune response to orally administered Sterne strain $34F_2$

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anthrax vaccine. Evaluation of serologic tests for diagnosis of anthrax after an outbreak was made by Harrison et al. (1989). Johnson-Winegar (1984) compared enzyme-linked immunosorbent with indirect hemagglutination assays for determining anthrax antibodies. A high-affinity monoclonal antibody to anthrax protective antigen passively protects rabbits before and after aerosolized *B. anthracis.* Spore challenge was studied by Mohamed et al. (2005). Though many studies have already been carried out on the disease and on the vaccines, no work has been performed using spectroscopic method and the present work aims to employ Fourier Transform Infra Red (FTIR) spectroscopic techniques to analyze the effect of Anthrax Spore Vaccine on sheep. A spectroscopic method of blood analysis is an alternate technique to the clinical methods since they require fewer samples and provide more information. In this work, normal healthy pre vaccinated blood samples (zero day) and post vaccinated (7^{th}, 14^{th}, and 21^{st} day of vaccination) blood samples were analyzed by employing FTIR spectroscopic techniques.

MATERIALS AND METHODS

Five healthy ovine female sheep were selected (weighing not less than 18 kg) as experimental animal; two were used as control and tested in the Institute of Veterinary Preventive Medicine (IVPM), Ranipet, Vellore District. Blood samples were collected from jugular vein of the experimental animal with necessary precautions. After collecting the blood samples (pre vaccinated or zero day), the sheep were vaccinated with Anthrax Spore Vaccine. Blood samples were collected from the same animal on 7^{th}, 14^{th} and 21^{st} day after vaccination. After collecting the blood, the sera samples were separated. Using the conventional method, the samples could be prepared by spreading a small volume of serum on an IR-transparent material, allowing drying and measuring the absorption spectrum of the film. The accuracy of the method may be compromised by any variation in the amount of serum successfully deposited on the KBr window, particularly with the manual sample preparation. In order to make up for this variation and to assess its impact on the overall accuracy of the method, a standard solution is added to each serum sample. The solution is chosen in such a way that it respond to IR radiation at the point where serum sample contains no absorption peak. Shaw et al. (1998) reported that the IR absorption spectrum of thiocyanate ion (SCN) includes absorption at 2060 cm^{-1} in a spectral region where sera samples subsequently normalize all of the spectra to equal intensities therefore compensating for the imprecision in the film preparation. A volume of 1 ml of serum was diluted with an equal volume of 4 mg/l aqueous potassium thiocyanate (KSCN) solution and 20 µl of each diluted sample was spread evenly over the surface of a circular KBr window (9mm diameter and 2mm thickness). Mid Infrared spectra in the region 4000-500 cm^{-1} were recorded on a "ABB BOMEM MB SERIES" – a FTIR spectrometer equipped with an air-cooled DTGS (Deuterated triglycine sulphate) detector. Strong absorption band of water in the mid IR region is hindered and to eliminate the same, the serum samples are air dried to form a thin uniform film on the KBr pellet. IR transparent KBr material without the samples is scanned as back-ground for each spectrum and 23 scans are co-added at a spectra resolution of 4 cm^{-1}. The collected signal is transferred to the PC. The data are processed by windows based data program – spectrum software. The spectra are base line corrected and they are normalized to acquire identical area under the curves and the maximum absorbance values of the corresponding characteristics bands are noted.

RESULTS AND DISCUSSION

The infrared spectrum provides various useful information of a biomolecule like structure, functional groups, types of bonds and its interactions. FTIR technique uses radiation wavelengths of the infrared region (2500-25000 nm) to excite vibrational levels of molecules. Infrared spectroscopy can be used to identify specific secondary structures. Each secondary structural element (that is, α-helix, β-sheet, β-turn and random coil) absorbs at specific wavelength regions (Pelton JT 2000). A satisfactory vibrational band assignment of the absorption bands of the spectra was done with the idea of the group frequency of the various constituents of the sera samples (Heise et al., 2008). The vibration band at 3304 cm^{-1} is due top the N-H stretching vibration of the secondary amides of protein. The asymmetric and symmetric stretching vibrations of the methyl group of proteins and lipids are found to be present at 2960 cm^{-1} respectively. The other two vibration bands in C-H stretching region are found to be present near 2936 and 2874 cm^{-1}, which are due to the asymmetric and symmetric stretching vibration of the methylene group. The strong absorption band present at 1660 cm^{-1} is attributed to c=o stretching of amide –I of the proteins. In the same way the presence of the band at 1545 cm^{-1} is due to the amide-II or NH bonding vibration that are strongly coupled to the C-N stretching vibrations of the protein amide groups. The peaks at 1457 cm^{-1} is considered to be due to the asymmetric and symmetric deformations of the methyl group of proteins. The spectra of pre and post-vaccinated sera samples were all distinct from one another, but were dominated mainly by the absorption of the protein constituent which provides the selectivity in infrared based serum analysis.

Table 1 presents the absorbance values for various wave numbers of pre and post vaccinated sheep. After the 21^{st} day of vaccination the sheep were challenged with virulent bacteria (that is, the live bacteria were injected into the animal body). The antibodies were produced as an anamnestic phenomenon consequent to 'memory' established while on primary vaccination. After the challenge test, the absorbance value of sheep numbers 1, 2 and 3 were given in different colours in the table and the animals were alive. Absorbance values for all the wave numbers in the post 3 (14^{th} day of vaccination) state were almost equal for sheep numbers 1, 2 and 3. For sheep 1 and 5, the variation is larger and hence the animal died after being challenged with virulent bacteria. This variation was expected due to the pre immune status of the animal. The postmortem reports showed that they died of Enteritis (loose motion and dehydration). Smear tests revealed that these animals were not affected by anthrax. By comparing Table 1 we

Table 1. Absorbance value for various wave numbers of pre and post vaccinated sheep.

Category	Days	3296	2960	2936	2874	1660	1545	1457
Sheep 1	pre	0.68	0.48	0.46	0.42	0.79	0.64	0.47
	post1	0.92	0.58	0.55	0.48	1.17	0.88	0.45
	post2	0.69	0.49	0.47	0.41	0.84	0.69	0.41
	post3	0.92	0.58	0.53	0.48	1.17	0.87	0.45
Sheep 2	pre	0.77	0.58	0.56	0.53	0.89	0.8	0.56
	post1	1.96	1.31	1.22	1.04	2.03	1.78	1.12
	post2	0.25	0.15	0.14	0.12	0.31	0.23	0.11
	post3	1.36	0.93	0.88	0.75	1.53	1.32	0.79
	cha	1	0.52	0.47	0.39	1.04	0.84	0.45
Sheep 3	pre	0.41	0.29	0.28	0.25	0.47	0.38	0.26
	post1	0.66	0.45	0.43	0.37	0.82	0.65	0.35
	post2	1.28	0.85	0.8	0.7	1.37	1.2	0.71
	post3	1.35	0.9	0.85	0.72	1.46	1.33	0.86
	cha	0.98	0.54	0.49	0.41	1.03	0.86	0.45
Sheep 4	pre	0.5	0.29	0.28	0.25	0.47	0.37	0.28
	post 1	0.14	0.09	0.08	0.07	0.18	0.12	0.06
	post 2	0.68	0.46	0.45	0.39	0.79	0.64	0.36
	post 3	1.23	0.83	0.78	0.65	1.37	1.25	0.78
	cha	0.93	0.56	0.52	0.43	1.06	0.85	0.41
Sheep 5	pre	1	0.45	0.43	0.4	0.82	26.1	31.9
	post 1	1.72	1.15	1.08	0.9	1.85	1.67	0.98
	post 2	0.9	0.63	0.6	0.53	0.98	0.82	0.52
	post 3	1.68	1.17	1.11	0.93	1.8	1.61	1.05

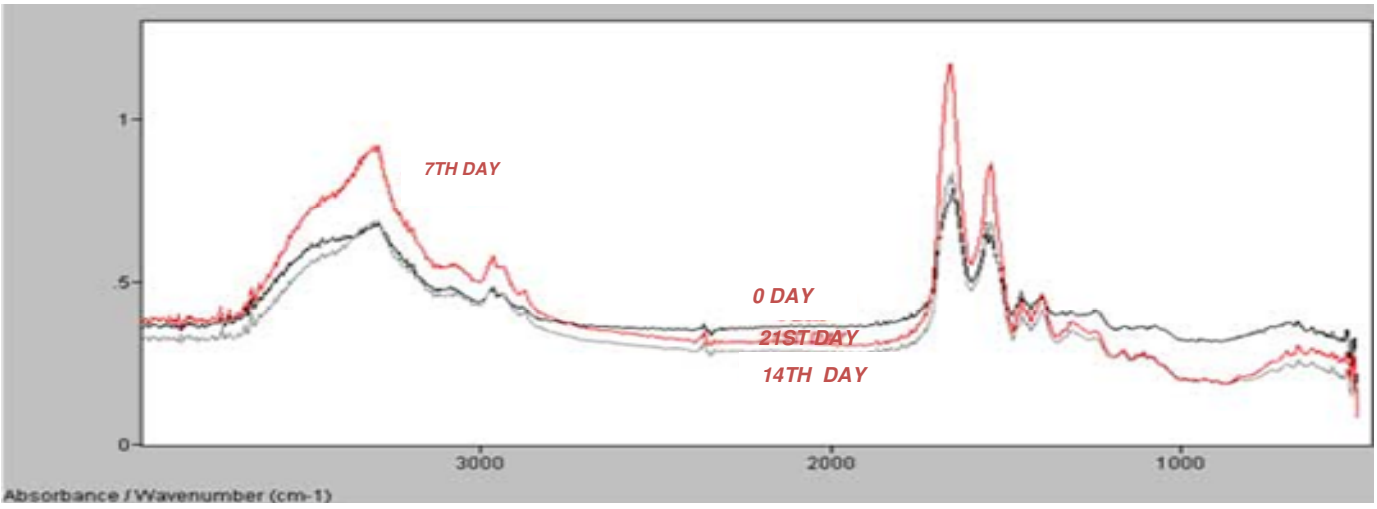

Figure 1. FTIR overlaid spectra of sheep 1.

can come to a conclusion that on the 21st day of vaccination (post 3) the absorbance value for all the animals were nearly equal, so those animals were alive after the challenge test. For sheep 1 the values were

Table 2. Descriptive table for the wave number 1660 for the sheep.

Category	Mean	S. D	S.E of Mean
Pre	0.689	0.204	0.091
Post 1	1.210	0.759	0.339
Post 2	0.858	0.382	0.171
Post 3	1.466	0.231	0.103

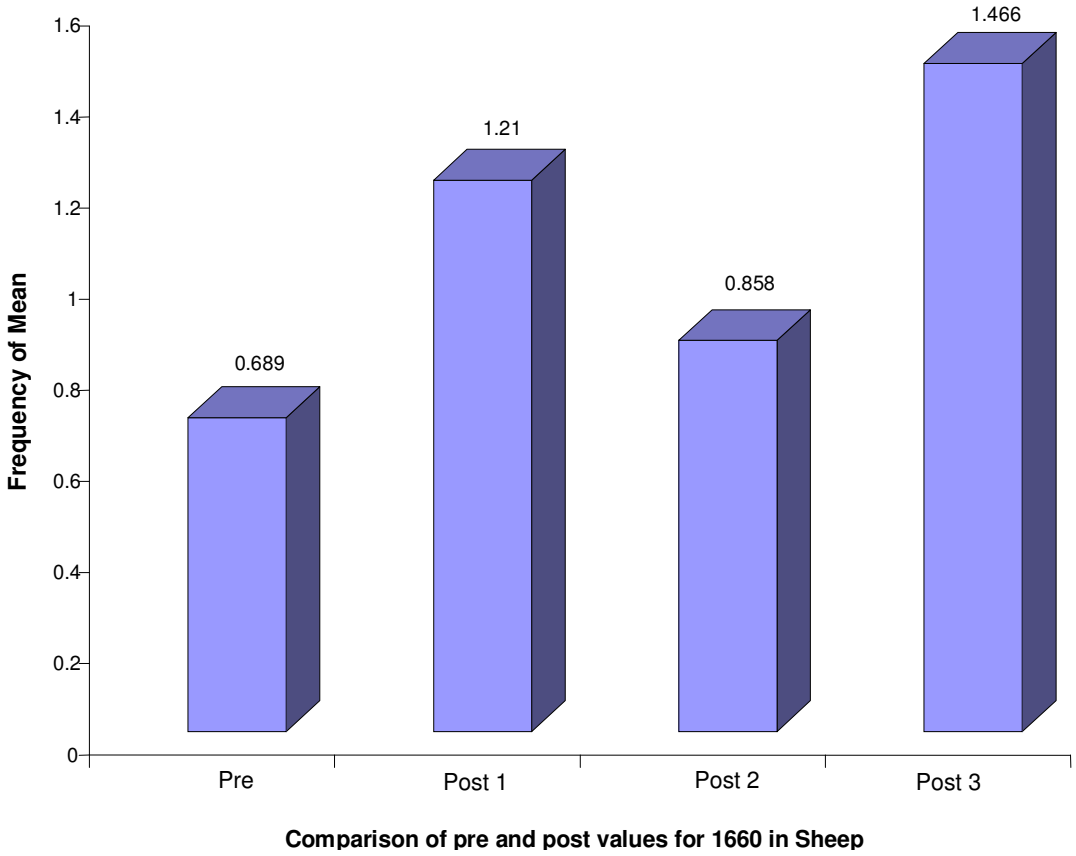

Figure 2. Bar graph for the frequency of the mean versus the pre and post states of sheep vaccinated with ASV for wave number 1660 which belongs to C=O stretching of amide –I of the proteins.

lesser and for sheep 5 the values were larger compared to the animals.

Figure 1 represents the FTIR overlaid graph of the pre (zero day), post 1(7^{th} day), post 2 (14^{th} day) and post 3 (21^{st} day) vaccinated states for sheep 1.

Table 2 represents the descriptive statistics such as mean, standard deviation (S.D) and standard error of mean (S.E of mean) for the sheep vaccinated with anthrax spore vaccine for the wave number 1660 belongs to c=o stretching of amide –I of the proteins.

Figure 2 shows the bar graph for the frequency of the mean versus the pre and post states of sheep vaccinated with ASV for wave number 1660 which belongs to c=o stretching of amide –I of the proteins. The mean value increased from pre to post 1, that is between zero to 7^{th} day of vaccination due to the antigen (vaccine) given to the animal. Between 7^{th} to 14^{th} day, the mean value decreased. From 14^{th} to 21^{st} day the mean value increased to maximum which denotes the maximum level of protein in the animal body due to vaccination.

From the Table 3 we have derived a highly significant difference ($p<0.05$) by using Analysis of Variance (F test), for the sheep vaccinated with anthrax spore vaccine for the wave number 1660 belonging to c=o stretching of amide –I of the proteins.

Table 4 represents the descriptive statistics such as mean, F value and P value for the sheep for various wave numbers. From the Table 4 we have derived no

Table 3. Anova table for the wave number 1660 for the sheep.

Category	Sum of squares	Mean sum of squares	F value	P value
Between groups	1.831	0.610	3.0	0.04
Within groups	3.266	0.204		

Table 4. Descriptive table for the various wave numbers for the sheep.

Wave number cm-1	Mean				F value	P value	significance
	Pre	Post 1	Post 2	Post 3			
3296	0.672	1.081	0.761	1.301	2.066	0.145	NS
2874	0.371	0.601	0.431	0.706	1.847	0.179	NS
2960	0.415	0.714	0.516	0.883	2.272	0.119	NS

Note: NS – No Significance variations.

significant difference ($p>0.05$) by using Analysis of Variance (F test), for the sheep vaccinated with anthrax spore vaccine for the wave numbers.

Therefore we can conclude that the anthrax spore vaccine given to the animal changed the protein level in the body in a highly significant manner. For the other wave numbers, it was observed that there was no significance variation between the pre to the various post levels of vaccination.

Conclusion

Animal tests play a crucial role in research and development (R&D) and in routine quality control of human vaccines. Quality control of vaccines, *in vivo* as well as *in vitro*, is important in all stages of vaccinology: (i) during R&D, (ii) during routine production and (iii) after production (batch release). The disadvantages of *in vivo* tests are numerous; they are expensive, inaccurate, slow and ethically questionable. Nevertheless, for many vaccines *in vivo* potency testing is performed on each batch. There is an urgent need for *in vitro* functional tests to determine the potency of the vaccines. If replacement of challenge tests turns out to be too ambitious, *in vitro* functional tests are attractive refinements. Confor-mational changes in proteins can be detected by a variety of spectroscopic techniques, such as circular dichroism, fluorescence spectroscopy and infrared spectroscopy (Bernard Metz, 2002). So this spectroscopic method can be confirmed with some other immunological tests in future as the best *in vitro* functional tests in the veterinary field of veterinary biologicals.

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