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Efficacy of Anthrax Spore Vaccine on Sheep: A Spectroscopic Approach

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In this work, normal healthy pre-vaccinated blood samples (zero day) and post vaccinated (7th, 14th and 21st day after vaccination) blood samples are analyzed by employing Fourier transform infrared spectroscopic techniques (FTIR). The variation in peaks is expected due to the production of antibodies in the animal. The internal standards among the application peaks are calculated. Among the various techniques to study the antibody production, ELISA (enzyme-linked immuno sorbent assay) is considered to be a better one, which can be done only in sophisticated laboratories. Spectral study can be taken as an alternate method and it can be compared with ELISA in future. Spectroscopic methods of blood analysis have an alternate technique to the clinical methods since they require fewer samples and provide more information.

Key Words: FTIR Spectroscopy, ELISA test, Anthrax spore vaccine, *Bacillus anthracis*.

INTRODUCTION

Anthrax is an infectious disease caused by the bacterium called *Bacillus anthracis*. Anthrax occurs naturally around the world in wild and domestic hoofed animal, especially cattle, sheep, goats, camels and antelopes. Anthrax is a disease of herbivorous animals caused by *Bacillus anthracis* and human incidentally acquire the disease by handling infected dead animals and their products[1-4]. Anthrax is usually spread in the form of a spore. Disease occurs when spores enter the body, multiply and release toxins. The incubation period of natural infection in animals is typically 3-7 days with a range of 1-14 days. In cattle and sheep, the per-acute course of illness may last only 1-2 h. The very first indication of problems may be sudden death of the animal. The inhalation of anthrax spores can lead to infection and disease. The possibility of creating aerosols containing anthrax spores has made *B. anthracis* a chosen weapon of bioterrorism. Several powers may have the ability to load spores of *B. anthracis* into weapons. Domestic terrorists may develop means to distribute spores *via* mass attacks or small scale attacks at local level. As an agent of biological warfare it is expected that a cloud of anthrax spore would be released at a strategic location to be inhaled by the individuals under attack. Spores

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of *B. anthracis* can be produced and stored in a dry form and remain viable for decades in storage or after release. Spores of *B. anthracis* can survive extremely long period of time in the environment. Survival time in soil, carcasses, textiles, water, sewage sludge and surfaces under open air conditions are of special interest in epidemiology. Survival times depend on the amount of exposed spores, temperature and a lot of other factors. Shakya *et al.*[5] studied the evaluation of immune response to orally administered Sterne strain $34F_2$ anthrax vaccine. 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 FTIR spectroscopic technique to analyze the efficacy of anthrax spore vaccine.

EXPERIMENTAL

Five healthy sheeps (each weighing not less than 18 kg), were under test in the Institute of Veterinary Preventive Medicine (IVPM), Ranipet. Blood samples were collected from jugular vein of the sheep. After collecting the blood samples (pre vaccinated or zero day), the animals (sheep 1-5) were vaccinated with ASV. Blood samples were collected from the same animals on 7th, 14th and 21st 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.*[6] reported that the IR absorption spectrum of thiocyanate ion (SCN) includes absorption at 2060 cm^{-1} in a spectral region where sera samples and subsequently normalizing all of the spectra to equal intensities therefore compensated 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 mL of each diluted sample was spread evenly over the surface of a circular KBr window (9 mm diameter and 2 mm 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. It has already mentioned that the 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 was scanned as back-ground for each spectrum and 23 scans were co added at a spectra resolution of 4 cm^{-1}. The collected signal was transferred to the PC. The data were processed by windows based data program-spectrum software. The spectra

were base line corrected and they were normalized to acquire identical area under the curves and the maximum absorbance values of the corresponding characteristics bands were noted.

RESULTS AND DISCUSSION

The spectra of pre and post-vaccinated sera samples are all distinct from one another, but are dominated mainly by the absorption of the protein constituent which provides the selectivity in infrared based serum analysis. A satisfactory vibration band assignment of the absorption bands of the spectra is done with the idea of the group frequency of the various constituents of the sera sample. Table-1 presents the vibration band assignment of serum. The vibration band at 3304 cm^{-1} is due to 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 2955 and 2869 cm^{-1}, respectively. The other two vibration bands in C-H stretching region are found to be present near 2936 and 2851 cm^{-1}, which are due to the asymmetric and symmetric stretching vibration of the methylene group. The strong absorption band present at 1656 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 1456 and 1315 cm^{-1} are considered to be due to the asymmetric and symmetric deformations of the methyl group of proteins. The peak at 1403 cm^{-1} may also considered due to COO^- stretching of ionized amino acid chains, suggesting an increased contribution from carboxalate. The lipid phosphate band due to the asymmetric PO_2 stretching vibration is found to occur at 1240 cm^{-1}. The spectral region 1169-1081 cm^{-1} is predominantly occupied by the C-O stretching vibrations of glucose. The absorption peaks present at 1169, 1153, 1107, 1079 and 1035 cm^{-1} are considered to be due to the different C-O stretching vibrations of C-O-H and C-O-C bonds. The weak absorption band at 955 cm^{-1} is considered to be due to PO_2 symmetric stretching of the phosphate bond of proteins. The medium strong vibration bond present at 625 cm^{-1} is assigned as N-H out-of-plane bending with the contribution of C-N torsional vibrations. The infrared spectrum provides various useful information of a biomolecule like structure, functional groups, types of bonds and its interactions. Hence the FTIR spectra of all the serum sample both pre and post vaccinated show the corresponding absorption bands in their specific regions qualitatively. But quantitatively there is a considerable spectral difference between the pre and post-vaccinated sera. The absorbance is directly proportional to the concentration. Hence the different sera samples are analyzed quantitatively by calculating the intensity ratio among the absorption peaks. In order to quantify the spectral difference, three intensity ratio parameters have been introduced. They are $R_1 = I_{3304}/I_{2955}$ due to the N-H stretching of secondary amides of proteins and the lipids, $R_2 = I_{1656}/I_{1545}$ which is due the ratio of intensities of amide-I and amide-II and $R_3 = I_{1403}/I_{1457}$ due to the ratio of the intensities of

COO^- stretching of amino acids CH_3 asymmetric deformation. Though the infrared spectra of all the sera samples were similar, considerable differences were found to be present in the internal standards among the absorption peaks in pre and post vaccinated samples.

TABLE-1
INFRARED VIBRATIONAL BAND FREQUENCY ASSIGNMENT OF SERUM

Vibration band (cm^{-1})	Assignments
3304	N-H stretching of secondary amides of protein: amide A
2955	CH_3 asymmetric stretching of proteins and lipids
2936	CH_2/CH stretching
2869	CH_3 symmetric stretching of proteins and lipids
2851	CH_2/CH Stretching
1656	C=O stretching (80 %) weakly coupled with C-N stretching (10 %) and NH deformation (10 %)-amide I
1545	NH deformation (60 %) strongly coupled with C-N stretching (40 %) amide II
1457	CH_3 asymmetric deformation
1403	CH_3 asymmetric deformation COO^- stretching of amino acids
1315	CH_3 symmetric deformation
1240	asymmetric PO_2 stretching of lipid phosphates
1169	C-O stretching
1128	C-O stretching
1081	C-O stretching
955	PO_2 symmetric stretching of lipid phosphates
701	NH asymmetric deformation coupled with CH_2 rocking amide V
625	O=C-N deformation (40 %) coupled with other ring deformation (60 %) amide IV

Table-2 summarizes the internal standard calculations of the pre and post-vaccinated samples of sheep. The R_1 value of sheep No 2 was 1.33 in the pre-vaccinated state but after vaccination the values increased till 14th day. During that period, the antibodies were produced in the animal body. On 21st day of vaccination the value decreased to 1.46, but it was greater than the value on zero day. After challenging the animal with virulent bacteria, the intensity ratio R_1 increased to a greater extent (1.92) and the animal was alive. But in the case of sheep No 5, the animal died after challenging with virulent bacteria. This was because of the R_1 value was 2.24 before vaccination. It was high compared to the other animal. After vaccination the value decreased continuously which means the spore vaccine given to the sheep neutralized the antibodies already existing in the animal. Even on 14th and 21st day it could not produces any antibodies. There was no change in R_1 value. R_2 and R_3 values were also varied drastically in sheep 5 compared to 2, 3 and 4. Sheep numbered 1 and 5 died after the challenge test. The postmortem reports showed that they were died because of Enteritis (loose motion and dehydration).

Even though it may be an accepted reason, by comparing Table-2 it is suggested that on the 21st day of vaccination the R_1 value should be 1.46 and above, so that animal can withstand for the challenge test.

TABLE-2
INTERNAL STANDARD CALCULATIONS OF THE PRE AND POST-VACCINATED SAMPLES OF SHEEP

Category	Day	$R_1 = I_{3304}/I_{2955}$	$R_2 = I_{1656}/I_{1545}$	$R_3 = I_{1403}/I_{1457}$
Sheep 1	Pre	1.41	1.16	0.97
	Post 1	1.58	1.41	1.04
	Post 2	1.42	1.26	1.02
	Post 3	1.59	1.35	1.04
Sheep 2	Pre	1.33	1.11	0.97
	Post 1	1.5	1.14	1.10
	Post 2	1.65	1.37	1.04
	Post 3	1.46	1.16	1.06
	Challenge	1.92	1.23	1.14
Sheep 3	Pre	1.44	1.23	1.00
	Post 1	1.48	1.32	1.02
	Post 2	1.51	1.14	1.04
	Post 3	1.49	1.10	1.05
	Challenge	1.83	1.20	1.14
Sheep 4	Pre	1.43	1.24	0.95
	Post 1	1.67	1.45	0.97
	Post 2	1.47	1.24	1.02
	Post 3	1.49	1.11	1.05
	Challenge	1.66	1.24	1.14
Sheep 5	Pre	2.24	0.04	1.02
	Post 1	1.5	1.11	1.08
	Post 2	1.43	1.16	1.02
	Post 3	1.43	1.12	1.08

Table-3 represents the mathematical analysis of P-value for various frequency levels. If the P-value varies from 0-0.01 two stars have been given which signifies the variation at 1 % level. If it varies from 0.011-0.050 single star has been given which signifies the variation at 5 % level. If it is more than 0.05 no stars have been given which signifies no variation. Between pre-post 1 state and post 1-post 2 state no stars have been given which denotes that there no significant variation occurs between 0-14 days of vaccination. From 14th-21st day of vaccination the immunity level increases in the animal body due to the production of antibodies. The difference between the pre to post 3 state is more. The maximum immunity level reaches on the 21st day of vaccination. Between post 3 to challenge state the variation is very high which denotes the production of antibodies against the virulent bacteria injected to the animal body on the 21st day of vaccination.

TABLE-3
P-VALUE FOR VARIOUS FREQUENCY LEVELS

Category	Pre-post 1	Post 1-post 2	Post 2-post 3	Post 3-challange	Pre-post 3
3296	0.191	0.505	0.043*	0.003**	0.005**
2960	0.173	0.537	0.055	0.014*	0.013*
2936	0.183	0.548	0.863	0.016*	0.017*
2874	0.213	0.564	0.069	0.017*	0.018*
1660	0.115	0.468	0.036*	0.017*	0.003**
1545	0.866	0.494	0.038*	0.003**	0.425
1457	0.493	0.545	0.038*	0.002**	0.754
1398	0.548	0.516	0.037*	0.020*	0.819
1315	0.315	0.540	0.043*	0.002**	0.037*
1240	0.398	0.552	0.045*	0.002**	0.051*
1169	0.543	0.593	0.048*	0.007**	0.077

Note: P Value 0-0.01 ** signifies at 1 % variation level, 0.011-0.050 * signifies at 5 % variation level, > 0.05 no star no significant variation.

Table-4 summarizes the internal standard ratio among the absorbance peaks in the FT-IR spectral analysis. R_1 represents the ratio between the protein and lipid level for the five sheep. R_2 represents the internal standard ratio between protein and amino acids. There is a considerable increase in the values of pre and post 3 for almost all the sheep. R_1 increases between pre and post 3 for 80 % of the sample taken. R_2 increases by 60 % of the sample taken. Thus it is concluded that the level of protein, lipid and amino acids are increase because of vaccination. The vaccine given to the animal increases its immunity level between 0-14th day of vaccination. On the 21st day of vaccination the immunity level reaches maximum.

TABLE-4
INTERNAL STANDARD RATIO AMONG THE ABSORBANCE PEAKS IN THE FT-IR SPECTRAL ANALYSIS

Category	Days	1660 Protein	1457 Lipids	$R_1 = I_{1660}/I_{1457}$	1660 Protein	1398 Amino acids	$R_2 = I_{1660}/I_{1398}$
Sheep 1	Pre	0.791	0.474	1.669	0.791	0.458	1.727
	Post 3	1.172	0.448	2.616	1.172	0.465	2.520
Sheep 2	Pre	0.892	0.564	1.582	0.892	0.494	1.806
	Post 3	1.533	0.446	3.437	1.533	0.414	3.703
Sheep 3	Pre	0.471	0.262	1.798	0.471	0.262	1.798
	Post 3	1.461	0.855	1.709	1.461	0.901	1.622
Sheep 4	Pre	0.468	0.275	1.702	0.468	0.262	1.786
	Post 3	1.365	0.782	1.746	1.365	0.822	1.661
Sheep 5	Pre	0.823	3.187	0.258	0.823	3.239	0.254
	Post 3	1.801	1.045	1.723	1.801	1.125	1.601

Conclusion

FTIR Spectroscopic method has been employed to study the pre and post vaccinated blood samples. The internal standard among the absorption peaks were calculated. By this technique the potency of vaccine as well as screening of antibodies level can be done. It can be used as an alternate test to *in vivo* test as CPCSEA- 'committee for the purpose of control and supervision on experimental animals' impose lots of regulations to use animals for challenge studies.

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