Pasteurella multocida is responsible for causing sporadic outbreaks of haemorrhagic septicaemia amongst cattle and buffaloes. In most Asian countries, it is recognised as a disease of utmost economic importance. It has a distinctive seasonal incidence, occurring soon after the onset of monsoon in certain low lying marshy and deltaic areas.

All countries where haemorrhagic septicaemia is enzootic, adopt vaccination as a preventive measure against the disease. Broth bacterins are most popular, but immunity amongst the vaccinated animals is of short duration. The oil adjuvant vaccine gives the longest immunity but finds use in only a few countries since it is thick and difficult to inject. Recent work on vaccine development has been directed towards producing thinner emulsions, isolating antigenic fractions and production of avirulent mutants for use as live vaccines. It is believed that a more effective control of the disease could be achieved with a better understanding of the epizootiology, production of better vaccines and by attaining a higher vaccination coverage.
In India, the most widely used vaccine against haemorrhagic septicaemia today is the alum precipitated vaccine. The main drawback is that the vaccine needs to be administered frequently, that is every six months, as the immunity rendered by it is not adequately long. For cross-bred and expensive animals, the haemorrhagic septicaemia oil adjuvant vaccine is employed. This vaccine is designed to retard absorption of vaccine and prolong the antigenic stimulus. As compared to other vaccines, it is considered to be superior in affording immunity. However, on account of its high viscosity and thick consistency the vaccine is cumbersome to administer when a large number of animals are to be injected at one time. Thus, in spite of this vaccine being highly potent, it has not gained much favour in the field because of these practical difficulties. Improvement and standardisation of the existing haemorrhagic septicaemia vaccine therefore appears to be a problem that deserves further attention, especially in our country.

As the first step, various media were tried in order to attain an abundant capsular growth of *P. multocida*. These included tryptose agar with and without glucose; tryptose agar with the addition of serum; starch dextrose agar; yeast extract agar with glucose, and heart infusion agar. The
criteria for assessment of growth was turbidity and capsular growth. The former was determined by employing Brown's opacity tubes. Capsular growth was assessed by staining the organisms by the Maneval's technique (1934).

It was observed that presence of glucose and serum in the medium changed the morphology of the organisms which was not desirable. Starch dextrose agar was a good growth yielding medium but would be too tedious to prepare on a large scale. Tryptose agar, too, gave good growth but was found to be expensive. Thus, Heart infusion agar which rendered abundant growth of capsulated organism with no change in morphology, was used routinely for all subsequent studies. An additional advantage is that the heart pieces are generally thrown off after preparation of Liver Robertson's medium. The same could be further utilised for the preparation of this medium.

In order to obtain capsular protein of a superior quality and maximum quantity, the crude extract was prepared from the bacterial suspension by three different methods - Maheshwaran method, Matsumoto method and the Penn and Nagy method. The only difference in the three methods was that in the latter the extract was obtained from live cells whereas in the first two, heat treatment was employed.
The Maheshwaran technique yielded the highest amount of protein from the crude extract. Secondly, the minimum protective protein dose of the crude extract prepared by the Maheshwaran method was determined to be the least, as compared to other methods. Thus the Maheshwaran technique was followed routinely for further studies.

In order to determine the relation between the amount of protein in a dose and the protection afforded, active immunisation was carried out in mice obtained from an inbred colony. The mice used in the entire study were about 4-5 weeks old and 18-22 gms in weight. Different concentrations of the protein, ranging from 1000 μg to 2 μg per ml of the crude extract, were injected subcutaneously in a group of 15 mice each and then challenged intraperitoneally on the 21st post inoculation day by a virulent culture of *P. multocida*. It was observed that the crude extract prepared by the Penn and Nagy method yielded a minimum protective dose of 15 μg/ml whereas in the CE prepared by the Maheshwaran method it was 10 μg/ml. Similar experiments were performed by Syuto and Matsumoto (1982) and Srivastava and Foster (1976), but they were not successful in establishing a good correlation between the dose and protection.

As the crude extract rendered 100% protection, the possibility of using it as a vaccine was explored by using two different adjuvants; alum and incomplete Freund's adjuvant.
The dose in cattle was calculated to be hundred times that of the mouse protective dose. Amies (1951) has presented some preliminary data to show that the crude preparations were good immunising agents.

The vaccine was tested for its sterility, safety and potency according to the P1 Schedule of the Drugs and Cosmetics Act, 1979. Six buffalo bulls were vaccinated with 1 mg protein of the CE and then challenged with a live 18 hour culture of the P 52 strain. The crude extract vaccine, both in IFA and alum, rendered 100% potency. Although this was a limited trial, the results were encouraging.

It was important to determine the duration of immunity rendered by the experimental vaccines. For this purpose two tests namely the passive mouse protection test and the indirect haemagglutination test were performed. The alum precipitated and the incomplete Freund's adjuvant vaccines prepared from the crude extract were injected intramuscularly in a group of six buffalo bulls each. Similar groups were also inoculated with the traditional alum precipitated and oil adjuvant vaccines. The animals were bled every month, post vaccination, for a period of 12 months and the serum separated and stored at -20°C until taken up for testing.
In case of the passive mouse protection test, it was observed that the traditional oil adjuvant vaccine rendered 60 per cent protection up to the 9th month and the experimental vaccine inIncomplete Freund's adjuvant up to the 8th month. The traditional and experimental alum precipitated vaccines gave 60 per cent protection in mice till the 4th month only, in the case of the former, and up to the 6th month in case of the latter vaccine. Roberts (1947), Bain (1963) and Vipulasiri et al. (1982) have shown that the passive mouse protection test is a very good in vivo test for assessing the humoral immunity in animals immunised against haemorrhagic septicaemia.

In the indirect haemagglutination test, it was observed that mice inoculated with the incomplete Freund's adjuvant vaccine prepared from crude extract, developed a titre of 1:160 during the 1st month, which progressively increased to reach a peak of 1:5120 in the 2nd month. Subsequently it came down to 1:1280, which persisted till the end of the 8th month. On the other hand, in the traditional oil adjuvant vaccine, the titre remained constant at 1:1280 till the end of the 8th month. Carter (1955) proved that the indirect haemagglutination test could be used to detect possible break down of immunity. Amies (1951) described it as a useful research tool for following the development of circulating antibodies in animals undergoing
active immunisation. A similar observation was made by Mukkur and Nilakantan (1969):

Thus it was found that the incomplete Freund's adjuvant vaccine gave satisfactory titres. Rao and Sambamurti (1971) have also proved that animals vaccinated with the incomplete Freund's adjuvant vaccines developed higher titres than those vaccinated with the oil adjuvant vaccine. There was thus a correlation between the results of the indirect haemagglutination test and the mouse protection test. A similar observation was made by Sinha and Prasad (1973), while working with a mixed haemorrhagic septicaemia and black quarter vaccine.

Since the crude extract and the vaccine derived from it gave satisfactory results, an attempt was made to purify the crude extract in order to determine whether a single protein fraction from it was responsible for the immunogenicity and the possibility of employing this fraction as a vaccine.

Purification was done by column chromatography using Sephadex G-200 for the gel filtration. It was observed that the crude extract separated into two peaks, designated as Peak I and Peak II. These two peaks were tested for the protein content by the Lowry's method and for their immunogenicity, in essentially the same manner as that for the crude extract. It was observed that only Peak I was immunogenic in mice. The minimum protective dose was 5 μg.
protein/ml only, whereas the cattle dose was calculated to be 500 μg/ml. At this stage, it may be pointed out that in the traditional vaccine production, the protein content (required as the minimum protective dose) is not calculated. The standardisation is carried out only by matching the whole cell suspension with tube No.7 of the Brown's opacity tubes.

Attempts were made to study the immunogenic value of the fractions as there is no data on vaccine produced from the crude extract and the purified peaks. The crude extract vaccines gave satisfactory results, hence a vaccine from Peak I was also prepared and tested. The vaccine was prepared in incomplete Freund's adjuvant. Its potency was tested in cattle on the same lines as the crude extract (as recommended in the F1 Schedule of the Drugs and Cosmetic Act, 1979). It was found to render 66% protection, which is considered as potent. The serum was collected on the 21st post inoculation day, just before challenge and tested in the passive mouse protection test and the indirect haemagglutination test to determine its titre. In the former test, 70% of the mice passively immunised with the above serum were protected and in the latter it had a titre of 1:640. Thus all the three test, namely, the direct challenge test, the mouse protection test and the indirect haemagglutination test were in consonance with each other.
The duration of immunity elicited by this vaccine was determined in mice by the active immunisation method. It was observed that the vaccine rendered adequate immunity for a period of 4-5 months. Trials need to be undertaken in cattle to confirm the results regarding the duration of immunity in larger animals.

The 2 peaks were biochemically analysed for their contents of phosphorus, uronic acid, hexose, heptose, glucose reducing sugar and nitrogen. The molecular weights were determined by the sodium dodecyl sulphate polyacrylamide gel electrophoresis method. This analysis would be useful as the basis for further detailed studies of the two peaks along with amino acid sequencing; which could be utilised in future for synthetic vaccine production.

The experimental vaccines produce immunity within 5-7 days after vaccination. Both these vaccines could therefore be used safely, in the face of an outbreak.

It was observed that the crude extract vaccine protected 60% mice against the challenge by a wild strain and the Peak I vaccine protected 70% of the mice, thus indicating the cross-protectivity nature of the vaccines. There was no deterioration in the quality of the vaccines at room temperature implying that they could safely be kept at room temperature for 3 months after production, without any appreciable loss in their potency. This is an additional
plus point of the experimental vaccines which could be used with advantage in a poor country like ours.

As regards the economics of production, it is seen that the cost of production is considerably reduced as demonstrated by the following data:

From the starting suspension of 105 ml quantity, a supernatant of 100 ml is obtained. From this, a maximum of 50 doses of the traditional oil adjuvant vaccine and 20 doses of the traditional alum precipitated vaccine can be obtained.

In the case of the experimental vaccines, from 100 ml of crude extract, 300 doses could be prepared of either the incomplete Freund's adjuvant or the alum precipitated vaccines. Regarding the Peak I vaccine, a total of 60 doses could be obtained from 100 ml of the suspension.

Therefore, when considered from all angles, it appears that the experimental vaccines are very promising. The only aspect that remains is carrying out extensive field trials with these vaccines to confirm the laboratory findings before they are employed commercially.