An Experimental Study on Combined Vaccines against Haemorrhagic Septicaemia and Black Quarter.

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Alakh Kumar Sinha.

An Experimental Study on Combined Vaccines against Haemorrhagic Septicaemia and Black Quarter.

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Alakh Kumar Singha, B. V. Sc. & A. H. (Ranchi).
Post-graduate Department of Bacteriology,
BIHAR VETERINARY COLLEGE,
PATNA.

C E R T I F I C A T E

This is to certify that the Thesis entitled "AN EXPERIMENTAL STUDY ON COMBINED VACCINES AGAINST HAEMORRHAGIC SEPTICAEMIA AND BLACK QUARTER" submitted for the degree of Master of Science (Vet.) in Bacteriology to the Magadh University by Shri Alakh Kumar Sinha embodies the result of his independent study which he has carried out under my supervision and guidance.

11.1.71.

(Lala B.M. Prasad),
Professor & Head,
Post-graduate Department of Bacteriology,
Bihar Veterinary College,
P A T N A.

A C K N O W L E D G E M E N T

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(A. K. SINHA)

C O N T E N T S

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C H A P T E R - I

INTRODUCTION.

INTRODUCTION

In India, Haemorrhagic septicaemia (H.S.) and Black quarter (B.Q.) constitute the major bacterial diseases of cattle and are responsible for heavy economic loss amounting to several millions rupees annually.

The farmers engaged in either agricultural operations or in dairy industry suffer a serious loss due to subjection of their animals to these deadly diseases in areas where they are endemic. These two diseases affect very adversely upon the economic resources of the country.

This loss in India due to H.S. alone is estimated to run to several lacs of rupees annually. The death toll on average is estimated to the tune of over 33,000 every year. Besides this, Past. multocida is responsible for losses in other species of the animals such as sheep, goat, swine, poultry etc.

The mortality due to B. Q. is nearly 25 to 30 percent among the cattle. It affects primarily young cattle and buffaloes in their prime of age. Sheep and swine also become victim occasionally to this disease.

In endemic areas, these two diseases have seasonal incidence and generally large numbers of out-breaks occur with the onset of monsoon. The onset of the disease is usually very sudden and the course is extremely short and rapid. Due to above reasons, the treatment with chemo-therapeutic agents and

antibiotics can hardly be taken up effectively in time. Thus, the prophylactic use of suitable vaccines remains the only tool for the control of these diseases in endemic areas.

The protection afforded by the vaccines presently employed against these diseases are not very lasting. The two vaccines are administered separately and are repeated annually. This involves considerable time and labour as well as the operational cost. Therefore, the need of a suitable combined vaccine which would confer dependable degree of immunity against Haemorrhagic septicaemia and Black quarter is keenly felt by the cattle owners and field veterinarians.

In human medicine, considerable success has been achieved in the control of deadly contagious diseases with the use of combined vaccines. In veterinary practice, however, no serious effect seems to have been made to evolve a combined vaccine against H.S. and B.Q. Kagan et al. (1959-1961) produced a combined vaccine against black leg, malignant cedema and pasteurellosis. They found it safe and effective in cattle and sheep against these infections. In India, Kausal and Kangude (1957) and Mohan and Nadis (1966) attempted to evolve a combined vaccine against H.S. and B.Q. They obtained encouraging results which warranted further investigation.

The present study was undertaken to investigate into the possibility of evolving a suitable combined vaccine against

H. S. and B. Q., which would afford adequate protection against these diseases in cattle. With this end in view, several modifications of whole cell culture of Past. multocida and Cl.chauvoei with and without adjuvants were tried. Their immune status in experimentally vaccinated animals were determined by challange and serological tests.

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C H A P T E R - II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

EPIDEMIOLOGY

1. Haemorrhagic septicaemia :

The epidemiology of H. S. in cattle is not very clearly understood. A large number of factors, apart of the causative bacterial agent, seems to be involved in the precipitation of clinical disease. Some of these factors such as the age and state of health of susceptible hosts, introduction of fresh cattle into the herd, carrier status among cattle, climatic conditions, various physical stresses, virulence of the specific etiological agent etc. have been particularly held to contribute to the extent and severity of the disease.

In Argentina, endemic disease of cattle, horse and sheep whose symptoms resembled H.S., was named pasteurellosis by Lignieres (Friedberger and Frohner, 1910), and another epidemic in buffaloes simulating H. S. in Italy was first described by Metaxa (1816).

With the onset of monsoon or winter rains, a large number of outbreaks apears in endemic areas. Weberd and Bimdrich (1934) observed in East and West Prussia that the districts showing highest mortality had a wave of infection in early summer, an abatement in late summer (August) and a further rise reaching the peak in November followed by rapid fall. In a field study

extended over 10 years, Vittoz et al. (1939) observed that (a) the first outbreak of pasteurellosis in buffaloes appeared following the first rain, (b) the disease in buffaloes continued up to rainy season, May - July being the worst period, (c) during the dry period, only sporadic cases occurred, (d) the outbreaks were more numerous when a period of mild but constant rain marked the transition between the dry and rainy season, (e) the annual incidence of pasteurellosis was associated with rain fall, humidity, density of air and the temperature. Rotov (1953) concluded that under humid and miled conditions, pasteurella organisms remain alive in rich soils, in thick herbage, in water and in buildings, while under dry conditions chances of their survival outside the host are negligible. Shamatava (1958), while discussing the environmental factors influencing the occurence of the disease stated that the causual organism retained its virulence in soil for 45 days in summer, 155 days in winter and 87 days in water in Georgia.

A statistical study of the occurrence of this disease during 1947 to 1962 in Bihar indicated that disease occurred more often during the period between May to September, with highest incidence in the month of July (Mohan and Hadis, 1966).

Pasteurellosis has been reported in various species of animals in different regions. This has been reported in sheep and cattle in Sudan (Curasson and Didier, 1932), in cattle in Norway (Hallesness, 1936), in pigs and buffaloes in Central

Anam (LeRoux and Gillion, 1939), in geese in a farm in Bengal (Mohan and Bhadury, 1947), fowl cholera in ducks in Hyderabad (Mohteda, 1947). Sen Gupta and Verma (1951) reported an account of an outbreak of pasteurellosis in swine.

Anon (1953) suggested that shipping fever in U.S.A. caused by pasteurella organisms should be termed pasteurollosis and not Haemorrhagic septicaemia. Carter and McShery (1955) suggested that consistant recovery of pasteurellae from the respiratory tract and its absence from the blood was pasteurollosis rather than Haemorrhagic septicaemia. The term Haemorrhagic septicaemia should only be used when referring to the acute septicaemic pasteurollosis of cattle produced by Robert type I of Past. multocida.

Bain (1957) reported that in susceptible cattle and buffaloes the disease is precipitated by various stresses and the first symptom was marked by profuse salivation. Pasteurellae were abundant in saliva and could survive in the open for 34 - 48 hours. Susceptible animals become infected from contaminated environment.

Nikiphorova (1958) reported that pasteurollosis occurred in cattle, buffaloes, sheep, goats, pigs, reindeer, horses, rabbits, wild boars, silver foxes, mink, fowls, geese, ducks, guinea fowls, turkeys and several species of wild birds in U.S.S.R. The strains isolated were Roberts type I or Carter type B. Type A predominated among avian. Ubeev (1958) observed

an outbreak of Past. septico infection in yoks, cattle and their hybrids in a mountaineous region of Moscow, where animals were subjected to high humidity and wide temperature variations. Kawah et al. (1960) reported that Haemorrhagic septicaemia causing heavy mortality among cattle in Iran was mainly due to Roberts Type I. Bain (1961) reported that in South-East, the disease was more prevelent in buffaloes than in cattle.

Studying the routes of infection in Haemorrhagic septicaemia in calves, Isanko and Vasilchenko (1937) observed that under normal conditions infection per OS was ineffective, but it occurred when the resistance of calves was lowered by keeping them in darkness. The disease can also be transmitted by fleese (Sapre, 1945). Amitrov et al. (1961) noticed that the disease wastransmitted from cow to calves via-milk. The older calves kept on milk substitute were not affected with the disease. Wells (1962), while working in the Ankole district of Uganda observed evidences to suggest that water-holes were the important means of spreading the disease and that the vagina may be a possible route of infection immediately after parturition.

An outbreak of H. S. reaches a peak with the infection of all susceptible animals and then gradually declines. Bain (1954c, 1955b) noticed natural immunity in about 10% of the cattle population in most Asian countries which was attributed to the existance of a type I strain of low virulence causing a benign immunising infection. Dhanda (1954) observed that such animals

may act to block further spread of infection in a population.

If no carrier animal survives, the disease may disappear from a village in 2 -3 years until infection is reintroduced. The chief reservoir of infection from season to season must be carrier animals (Bain, 1954c, 1955b, Dhanda, 1954). On exmining nasal swabs from living and dead animals, Singh (1948) isolated pasteurella organisms from 3.5% of living and 7% dead animals. However, the organismswere not typed. Eleven out of fifteen strains of Past. multocida isolated by Singh (1948) were typed later by agglutination tests as type I of low virulence. This carrier stage of the animal in the respiratory passage of the animals may be imparting naturally acquired active immunity to the animals. The first case of the disease may be the result of clinical breakdown of these carrier animals as a result of exhaustion or inclement weather (Dhanda, 1958).

Omar et al. (1962) reported that a virulent and antigenic strain of Past. multocida was isolated from the tonsils of an apparently healthy buffalo. This isolation, probably the first of its kind, tends support the hypothesis that an arrested infection is the cause of the naturally acquired immunity to Haemorrhagic septicaemia in cattle and buffaloes.

Perreau et al. (1964) emphasised the importance of natural acquired immunity. Haemorrhagic septicaemia was rare and was very little known in Chad with the possible exception of the "Mayokebbi" region. However, mouse protection test revealed serum

antibodies in 82 out of 411 adult zebu slaughtered cattle. The sera were also tested by a passive haemagglutination test, using human group "O" erythrocytes.

Nangyalai (1969) isolated Past. multocida from 4.9% of healthy calves. The strains were highly virulent and killed rabbits within 18 - 36 hours.

2. Black quarter :

Clostridium chauvoei was first described by Bollinger and Fesser (1876, 1878), and Arloing et al. (1887). These authors reported that the disease could be transmitted to cattle by inoculation of infective materials. From the lesions, they isolated an anaerobic spore-bearing bacillus -Cl. chauvoei.

In India as well as in other countries, Black quarter or Blackleg is widely prevalent. The disease affects primarily young cattle in prime condition, but young buffaloes and sheep are frequently affected (Iyer and Ramahandran, 1954). The swine are affected less often (Meyer, 1915). The infection is caused by Clostridium chauvoei. The pastures become infected with the resistant spores of the causative organisms due to improper disposal of carcasses. The spores remain alive in the soil for long time. Kovalenko (1956) reported that in loamy soil, Cl. chauvoei remained alive for 16 months, whether at surface level or 15 cm deep and this was the main source of infection in endemic areas during the rainy seasons.

Black quarter in cattle is generally caused by Cl. chauvoei, and in sheep by Cl. septicum (Beller, 1920, Weinberg and Mihailesco, 1929).

In India, nearly 90% of the cases of Black quarter are reported to be caused by Cl. chauvoei. The remaining cases result due to infection with either Cl. septicum alone or in association with Cl. chauvoei (Iyer, 1952). Earlier, Viswanathan, (1934) had reported by serological procedure the presence of Cl. chauvoei and Cl. septicum in outbreaks of B. Q. in Madras State. However, Cl. welchii and others causing similar infections as stated by Gaiger (1924), Lechlainche and Valle (1925) and Naidu (1937) have not been encountered in Madras state of India so far.

The extensive investigations carried out in New Zealand and summarized by Buddle (1954) incriminated Cl.chauvoei as the sole aetiological agent.

All over the world, Black quarter is essentially an endemic disease with a seasonal and regional distribution causing heavy recurrent loss in particular areas. Its true distribution today is obsecured by the widespread practice of vaccination. However, it is true that the disease in cattle is of endemic nature and the infective agent in the soil is maintained and concentrated by pasturing of cattle and sheep. Even in warmer climates, it is recognized as essentially a disease of permanent pasture (Robinson, 1946).

The available data regarding the incidence of Black quarter in India indicate that the disease occurs in endemic form frequently during May to September every year, and the outbreak begins soon following the onset of the monsoon. Iyer and Ramchandran (1954) reported that most of the interior districts of the Madras State are endemic areas for Black quarter and there the disease occurs year after year just after the onset of the monsoon rains. Viswanathan (1934) also reported outbreaks of Black quarter in cattle in Madras States during rainy seasons.

A statistical study of the occurrence of this disease in Bihar during 1947 to 1962 indicated that the disease occurred more often during the period between May to September, the peak incidence being in the month of June and July (Mohan and Hadis, 1966). The study also revealed the endemic areas of this disease. This helped in planning the control programme by timely vaccination in endemic areas.

In Great Britain, the parturient gas gangrene in sheep was reported to be confined in particular flocks depending on the nature of the terrain. The disease was more frequent during the lambing season. It was suspected that increased cantamination of shepher's hands contributed to this condition but there was no rationship between the distribution of the disease in cattle and sheep.

Newsom and Cross (1933) reported the outbreak of

blackleg in a herd of sheep in Colorodo due to which about 25 ewes developed infection of the uterus with Cl. chauvoei.

Black quarter in sheep following vaccination, shearing, tailing and docking occurs as sporadic outbreaks, but its incidence in these circumstances is not significant on any one farm over a period of years.

CONTROL OF THE DISEASES

Agriculture and livestock wealth form the back bone of Indian economy and for successful livestock industry it is necessary that the livestock must be kept free from diseases. During the recent years, control of contageous diseases of animals has attracted much attentions of national and international bodies who are now actively engaged in this direction with commendable success.

The control of diseases depends to a large extent on a clear understanding of the aetiology and epizootiology of the disease. Based on epidemiological observations and accurate diagnosis of an epidemic, suitable control measures can be effectively undertaken. The use of effective vaccine constitutes an important step in the control of any contageous disease.

1. Haemorrhagic septicaemia : Live vaccine :-

In endemic areas, control of Haemorrhagic septicaemia lies in the effective prophylaxis of susceptible animals. In order to achieve measurable success in this direction, it is necessary to have at hand vaccine of good quality which can be easily made and can produce lasting immunity from a single injection of small volume. Previously, it was considered that in India pleurality of strains might be a factor affecting the production of a good vaccine. However, Bain (1954b), Hudson (1954),

Dhanda et al. (1956) and other workers reported that Past. multocida type I was the cause of Haemorrhagic septicaemia in majority of cases. The difference in strains did not make the preparation of an efficient vaccine against Haemorrhagic septicaemia in Asia and East Africa problematical.

The attenuated strains were used for protecting cattle since the days of Pasteur. On working with virulent and avirulent strains of Past. multocida, Hadley (1913) found that a high order of immunity could be obtained in rabbits vaccinated with avirulent cultures. These rabbits with-stood challenge against virulent strains. Hardenbergh and Boerner (1916, 1917, 1918), could not attenuate their strains by incubating cultures at 42.5°C for 5 days. Surprisingly, subcutaneous inoculation of 1 - 2 ml. of this culture caused only a slight local swelling in calves of 2 - 3 months age. On the basis of these findings, these workers used living vaccine prepared with a 48 hour old culture in glycerin broth. With such treatment, only 5 animals died of H.S. out of 434 animals treated. Subsequently, large numbers of animals were vaccinated and the results were quite satisfactory.

In India, Doyle (1923) attenuated a strain of Past. multocida in rabbits.

Basset (1935) found that greater the virulence of the seed culture employed in vaccine, more solid was the immunity produced. He observed that by growing Past. multocida first on weak agar medium for two months and then sub-culturing on this

medium for 4 successive passages at two months interval, the strain became attenuated. The vaccine retained its antigenic property for one year.

Otten (1939) used a living vaccine and showed that smooth strains gave better protection than those which were rough.

Carter (1950) prepared Past. septica vaccine in chicken embryo. It protected mice against more than 1,700 lethal doses of Past. septica. The most immunoganic broth bacterin conferred protection not in excess of 6 L. D. of the same challange culture. Subsequently Carter (1951) observed that mice immunised with chicken embryo vaccine prepared from types A, B & C of Past. septica demonstrated a strong resistance to homologous challenge, but displayed only a low grade of immunity against heterologous challenge infections.

Pegreffi et al. (1951) prepared vaccine by exposing Past. multocida to streptomycin. They reported that the antigenic activity of the streptomycin-treated vaccine resided in the organisms themselves, which were not lost when they were merely devitalised by the antibiotic. Mura and Contini (1951) prepared a similar product but used penicillin instead of streptomycin. They observed marked lymphocytosis in rabbits innoculated with the penicillin-devitilized vaccine. This lymphocytosin did not occur when a vaccine not so treated was used.

Hudson (1954) found that it was difficult to protect rabbits and mice with dead vaccines. He observed that the rabbits could be protected against small doses of virulent organisms of the same strain by repeated administration of vaccine prepared from heat or formalin killed broth culture, but such rabbits would at times succumb if larger doses of virulent organisms were given 10 - 14 days later. This observation made him to switch on to live vaccine prepared by selecting colonies of reduced virulence from plates inoculated with old cultures. He concluded that immunity in mice varied directly with the virulence of the strain used in the vaccine. Late on, he selected a strain of medium virulence (Past. 4/3) and induced artificial mutation by repeated sub-cultivation in aerated broth cultures and plating the growth on solid media. He thereby obtained colonies of varying grades of virulence. With such colonies, he prepared a liophylised vaccines which in Thiland protected 13 out of 16 buffaloes against a dose of virulent organisms which killed both the controls.

Rajagopalan (unpublished observation), while comparing the colony characters of virulent and avirulent strains by pour plate method, found that epidemic strains gave rise to lenticular opaque colonies in the depth of the medium but relatively large opaque low convex greyish-white colonies at the surface. At 48 hours, the colonies measured 2.5 to 4.7 mm. in diameter. The avirulent strain under the same condition produced slightly raised translucent bluish surface colonies of about the same size. The

deep colonies were indistinguishable from those of the virulent strains. The virulent colonies were fluorescent while avirulent ones were blue.

Dhanda et al. (1956) passaged virulent strains of Past. septica ($P_{52, 50 \& 5}$) for over two years in the developing chicken embryo and on different media, i.e. plain agar with different pH values, bile agar and pencillin-agar in order to see if any of these treatments would reduce their virulence and render them suitable for vaccine production. Only one strain i.e. the bile passaged strain P_{52} was found to lose virulence after it had undergone as many as 384 passages. However, it showed evidence of loss of its antigenicity. They also passaged a virulent strain of Past. septica in albino ratswhich were normally resistant to this organism, in the hope that the resulting attenuated strain might yield a good immunising antigen for vaccination against H. S. After 31 serial passages, carried out by transfer of blood from one rat to another, the cultures obtained at 5, 10, 15, 20 and 31st passages were tested for virulence in white rats, guinea-pigs and white mice. The strain was found to have assumed enhanced virulence for rats and guinea-pigs but they found no evidence of attenuation of this strain for white mice.

Shirlaw (1957) reported high degree of immunity with a vaccine prepared from a rough avirulent Kenya strain of Past. boviseptica but it was not possible to maintain its rough characters through successive cultures. He felt that a vaccine of such

a character would be useful in protecting cattle against infection. He further added that if rough characters were maintained, cattle could be immunised with a small dose of such a product effectively at a lower cost of production.

Bain (1957) suggested that the natural active immunity found in about 10% of cattle population in most Asian countries was due to existence of type I strain of low virulence which had resulted in a benign immunising infection. He stressed that if such a strain were found, it might prove to be a good antigen for successful immunisation against H. S.

Killed vaccine:

A better vaccine is the one which, even after the process of killing, preserves the protective antigens of the organism intact.

The majority of commercial vaccines prepared for the protection of cattle and buffaloes against H. S. contain organisms killed by heat or formalin. Formalin has been used till date universally in the preparation of pasteurella vaccine. Knox and Bain (1960) found that it binds the protein and polysaccharide components of type I Past. multocida so that they cannot be separated by usual methods. This may explain the beneficial effect of formal-killed vaccines and also the inagglutinability of type I suspensions by anti-serum (Bain, 1954b).

Tultschinskaja-Tkalzchenko (1934) prepared a vaccine by adding crystal vilet (1: 5000) to a broth culture of Past. multocida and found crystal violet to be superior to methylene blue (1: 20,000) and malchite green (1: 20,000) for this purpose.

Rajgopalan (1944, unpublished observation), confirmed the presence of capsules in type I strains of Past. septica and stressed that surface antigens must be preserved in the vaccines to be effective. Bain (1954c) described in detail the immunogenic state of type I strains under the provisional term 'Phase I' and suggested the necessity of monthly passage of the strains in buffaloes calves of cattle for their maintenance in complete antigenic phase.

Rajagopalan (1941, 1942) made extensive studies on the capsules of Past. septica and then susceptibility to different disinfectents. He observed that (a) virulent and avirulent strains were distinguishable by their cultural characteristics. The former formed larger, relatively opaque and fluorescent colonies with larger repulsion zones in India ink preparation but the latter gave rise to relatively small irridesent colonies. Morphologically, virulent cultures were predominantly coccoid in shape but the avirulent strains varied in being bacillary and non-encapsulated forms, (b) a suspension of Past. septica in a concentration of 3000×10^{-6} organisms per ml. in phosphate buffer

(pH 7.6) was completely sterilized when exposed for 5 minutes to 37°C, lysol (0.11%), merthiolate (1 : 22500), alcohal (12%) and formalin (0.5%), (c) that lysol and merthiolate significantly inpaired immunogenicity of the vaccine but alcohal and formaline did not, (d) that vaccines made from broth cultures and sterilized by heat (56°/ 1/2 hour) or disinfectants were inferior to vaccines similarly treated but prepared from growth in agar slants washed in buffer phosphate. If, however, the broth culture was washed and resuspended in phosphate buffer before sterilization, the loss in potency did not occur. It appeared that the immunizing antigens were impaired when sterilized by heat or disinfectants, the organisms being in contact with foreign proteins (e) a vaccine made from a 24 hour agar washed culture was as potent as one of the same strength from a 6 hour agar culture, (f) avirulent strains, even when living, did not immunize as efficiently as the virulent strains and (g) it was highly important to ensure that the structure of the essential part of the germ, particularly their surface layers did not suffer by the treatment adopted to kill them. These observations formed the basic foundation of the modern concept of vaccine production, which lateron came in the words of Bain, Dhanda, and Carter in their several papers.

Rau and Govil (1950) reported that H.S. agar-washed vaccine was better than broth vaccine. The higher the concentration of the vaccine, the stronger the immunity. An

agar-washed vaccine, formalised or heat treated, gave similar protection. Immunity produced by such vaccine of apacity 2 x 10 persisted upto 4 months after vaccination.

Haq and Quader (1954) prepared Past. septica vaccines from sero-fibrinous fluid collected from local oedematous lesions of H. S. by additing to it 1% crystal violet and 1% formaldehyde. A dose of 5 ml. of this vaccine protected 6 cattle against subsequent challenge. Haq and Quader (1957) later prepared a satisfactory vaccine by mixing equal parts of sero-fibrinous fluid and a washed suspension of 24 hours old agar culture inactivated by the addition of 0.5% formalin. It protected all of 38 cattle for at least 9 months.

Adjuvant vaccines :-

Nikiforva (1953) prepared an alum-precipitated vaccine from immunogenic strains of pasteurella organisms obtained from cattle, sheep and pigs. On farms infected with pasteurella, 140,000 cattle, sheep and pigs were given two injections of the vaccine at an interval of 10 - 20 days. In another field trial, 20,000 cattle were given a single dose of the vaccine. In both the cases, the animals were immune to pasteurollosis for at least six months following vaccination.

Suchaci et al. (1956) noted that addition of aluminium hydroxide gel to cultures of pasteurellae improved its immunogenic

quantities. They however, observed that this vaccine could be further improved by killing the organisms by 2.5% formal saline and concentrated by removal of the supernatant broth.

In 1952, Bain isolated "Insein" strain of Past. multocida in Burma and this was incorporated by Bain and Jones (1955) for the preparation of oil adjuvant vaccine. The advantage with this strain was that it remained in fluorescent phase for several subcultivations after recovery from the experimental host and all its antigenic characters could be maintained for comparatively longer period. This eliminated the difficulty of maintaining the strain in phase I. For preparation of vaccine, Bain and Jones used both solid agar and broth media. The difficulty of obtaining dense bacterial suspension was overcome by aeration of the culture (Bain and Jones, 1958). The density of broth or washed culture suspension was kept at 7 Brown's opacity tube. For formol-killed agar washed bacterial suspension, the oil adjuvant was used in the following proportions :-

Bacterial suspension	-	15 parts
Oil	-	10 parts
Lanolin	-	1.5 parts

For the broth grown suspension :-

Killed broth culture	-	15 parts
Oil.	-	12 parts
Lanolin	-	2 parts.

From aerated broth, 500 doses per litre of adjuvant vaccine could be obtained provided the density was adequate. Dhanda et al. (1956) also prepared an oil adjuvant vaccine of Past. multocida type I (P_{52}) by using slightly different solid media (Yeast extract-creatinin-agar). They used 1% lanolin instead of 1.5% of Bain and Jones (1955).

Iyer et al. (1955) and Iyer and Rao (1959) compared the immunising effect of oil-adjuvant vaccine with alum-precipitated vaccine in buffaloes. They found alum-precipitated vaccine to be inferior to oil-adjuvant vaccine, but the former confirmed a better and lasting immunity than the killed cultures without alum. These authors stated that since alum vaccine could be prepared and injected more easily than the oily vaccine and the latter necessitated the use of expensive manufacturing equipments, alum-precipitated vaccine may be given preference.

Bain (1954b), in a series of experiments, reported that the adjuvant phase I vaccine could protect 36 of 39 cattle and buffaloes in Burma and Siam against 25,000 and 200,000 lethal doses respectively of the homologous organisms injected subcutanousally 4 -5 weeks after vaccination. Bain (1956) further extended his investigation with varying doses (2 ml. and 3 ml.) of oil-adjuvant vaccine. Cattle and buffaloe immunized with 3 ml. dose of the vaccine survived challange 6 months later. This dose induced in cattle the formation of mouse protection antibody 11 months

after vaccination.

Bain (1957) observed that duration of the immunity produced by a single dose of up to 10 ml. of plain bacterin was 1 - 2 months but this could be enhanced with alum-precipitated or oil adjuvant vaccines.

Dhanda et al. (1958) carried out extensive studies on the keeping quality of the adjuvant vaccine by exposing the same at different temperatures for varying periods of time. The vaccine was found to be potent when kept at low temperature in dark. It did not deteriorate in its protective power for at least one year. It could withstand fairly high temperature and could be safely transported by rail or road under ordinary conditions to ate distant places covering a travelling periods of 10 days during rred summer months.

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Kaweh et al. (1960) reported good results with the e of Past. vaccine consisting of an autolysate of organisms obtained by tex tank. incubating the bacterial suspension in thimersolate solution to o. II and which was added saponin afterwards. The duration of immunity or 18 - 20 produced was 1 year. This vaccine was used very extensively in uvant,

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after vaccination.

Bain (1957) observed that duration of the immunity produced by a single dose of up to 10 ml. of plain bacterin was 1 - 2 months but this could be enhanced with alum-precipitated or oil adjuvant vaccines.

Dhanda et al. (1958) carried out extensive studies on the keeping quality of the adjuvant vaccine by exposing the same at different temperatures for varying periods of time. The vaccine was found to be potent when kept at low temperature in dark. It did not deteriorate in its protective power for at least one year. It could withstand fairly high temperature and could be safely transported by rail or road under ordinary conditions to distant places covering a travelling periods of 10 days during summer months.

Perrean (1960) reported that alum-precipitated vaccine conferred early and satisfactory immunity, the duration of which was not less than 6 months. This period was sufficient to protect cattle during rainy season. Perrean also considered that adjuvants prolonged the immunity.

Kaweh et al. (1960) reported good results with the vaccine consisting of an autolysate of organisms obtained by incubating the bacterial suspension in thimersolate solution to which was added saponin afterwards. The duration of immunity produced was 1 year. This vaccine was used very extensively in Iran

after 1959 and helped in the control of the disease there where the disease now occurs only sporadically. They suggested that saponin was a better adjuvant than either alum or oil.

In India, Rao and Shanmugham (1964) prepared an alum-precipitated vaccine. The supernatant was discarded. The sediment containing the bacterial precipitate proved to be an efficient antigen provoking a substantial immunity when used in as small a dose as 0.5 ml. subcutaneously in buffalo calves.

In India, Nangia et al. (1966) prepared a few batches of oil adjuvant vaccine from the bacterial suspension grown on yeast extract-agar medium without creatinin and substituting simple meat extract for lab-lemco. It was found to give adequate immunity in hill bulls or buffaloes calves. The vaccine conferred immunity in cattle even after storing at 45°F. for 814 days or on being subjected to 37°C. or 42°C. for 20 days. The immunity after a single injection lasted for 850 days (the longest period tested). Further, they stated that rabbits can conveniently replace hill bulls or buffalo calves for assaying the potency of H.S. oil-adjuvant vaccine.

Geneidy et al. (1967) prepared a dense culture of Past. multocida (Roberts type I) by aeration in a Bain's vortex tank. The medium employed was a combination of Bains medium No. II and Stern's pancreatic auto-digest and incubated at 37°C. for 18 - 20 hours. Formalin was added to give a final 0.5%. For adjuvant,

water in oil emulsion was prepared in a turbo-emulsifier, using 25 parts bacerial suspension, 20 parts "Risella 17" oil and 4 parts of lanolin (croda). Stable emulsion were produced, which improved after a few days of ripening. This could be stored and distributed under ordinary field conditions.

In India, Mukkur and Nilakantan (1968) compared the efficacy of the sodium alginate and oil adjuvant Past.multocida vaccines. Formalised Past. multocida suspension (1 -9 million cells) were mixed in equal volumes of 1% sodium alignate or oil adjuvant. Determination of the H. A. titres in six cattle 7 - 69 days after vaccination showed that the oil adjuvant vaccine produced slightly higher average titres than sodium alginate one.

Thomas (1968) obtained dense culture of Past. multocida by intermittently aerating and churning the media. Incubation of cultures for more than 24 hours did not increase the density of the cultures. The emulsified vaccine proved very stable under varying storage conditions.

From the above observations, it can be seen that the plain bacterin in a single dose up to 10 ml. produces less dependable immunity than alum-precipitated or oil-adjuvant vaccine used in moderate doses. The oil-adjuvant vaccine has been preferred to others but owing to high cost of production and lengthy procedure of manufacture, this has not yet been taken for use universally for the control of out-break of H. S. in the field.

2. Black quarter :-

During the past two or three decades, different prophylactic agents have been used to control the outbreaks of black quarter successfully. Immunization of cattle against black quarter was started as early as 1887, when Arloing et al. (1887) demonstrated the utility of heated powdered muscles obtained from infected animals, known as 'Arloing vaccine or the Lyons vaccine'. This method or its modification remained in vogue for a little over three decades. Later on, with the observation of Roux (1888) that guinea pigs could be immunised by means of filtered exudates or filtrate obtained from broth cultures of Cl. chauvoei and from further extension of the work by Nitta (1918) the use of natural and artificialaggressins came into existence.

Reichel and Schneider (1932) prepared a potent Blackleg aggressin by using a 48 hour blackleg culture grown in liver brain medium. The infective dose for bovine awas found to be 1.5 cc. and for sheep 0.075 cc. These authors reported that 0.5% formaline was preferable to 0.5% phenol as presurvative for aggressin.

Workers had been planning to change over to culture vaccine many years earlier, but the second world war delayed their fruition. After second world war, a whole culture bacterin containing dead bacterial cells together with the products of their metabolism was reported to be efficacious by many workers and these products yielded very promising results.

Because of the inherent difficulty in the preparation of aggressin, formalised whole culture vaccine was introduced by Lechlainche and Valle (1925), McEwen (1926), Karman (1927) and others. Thus, they developed an effective agent for the control of Black quarter and the hazards of vaccinations met with the use of other products were reduced to the minimum. This method is widely practiced in the various countries of the world and recognised as a standard, practical and safe method of immunisation against Black quarter. The immunity established is both anti-toxic and anti-bacterial and is of a high order.

Henderson (1932) and Mason (1934) demonstrated that the immunity in Black quarter was primarily due to the sometic antigen in bacterial cells. Later, Mason (1936) announced that Cl. septicum 'toxoid' could confer protection in sheep against both Cl. chauvoei and Cl. septicum infections. Knowledge about these facts led to the evolution of Cl. chauvoei-septicum bacterins. Since the quality of immunity conferred was reported to be dependent chiefly upon the concentration of bacterial cells in the vaccine, continued attempts were made to secure increased growth of Cl. chauvoei and Cl. septicum in nutrient media (Rajgopalan, 1947, Iyer and Ramchandran, 1954, Nilakantan, 1965).

VanSaceghem (1938) reported that in Belegian Congo Veterinary laboratory, black quarter vaccine was prepared from a virulent strain of Cl. chauvoei in liver broth medium, layered

with vaseline. After good growth had occurred, 0.5% formalin was added. When it was found sterile, this was issued as vaccine. The dose was 10 cc. and he claimed that the duration of immunity produced by this vaccine was at least 6 months. Martine (1938) suggested the addition of 0.1% glucose in the above medium which gave good growth. He inoculated 5-liter flasks with a portion of muscles from Guinea pig just dead from Cl. chouvoei infection. After 1 - 4 days incubation, the culture was killed by the addition of 0.6% formalin. This killed culture was issued as vaccine and was found to be effective.

In India, Haddow (1933) introduced the use of formalised whole culture vaccine, based on the belief that more than one members of the clostridium group are responsible for black quarter. Black quarter vaccines were prepared using Cl. chauvoei and Cl. septicum in many of the biological product centres in the country. The medium used for growing the organism generally consisted of nutrient broth to which tissue particles or fresh rabbit kidney were added to create anaerobiasis. Mixed cultures of organisms grown for 3 and 14 days were used. Rajagopalan (1947) reported on the utility of tissue-free media, viz Cysteine hydrochloride broth and acid digest broth for growing Cl. chauvoei. Verma et al. (1953) indicated that Black quarter vaccine could be prepared at a lesser cost by using papain digest broth.

Scheuber (1944) reported that alum-precipated vaccine

gave marked protection against infection, though such vaccine produced some local reaction. Sterne et al. (1951) produced Black quarter vaccine by growing Cl. chauvoei in cellophane bags in specially designed apparatus.

Piercy (1951) compared the efficacy of muscle, whole culture and alum-precipitated Black quarter vaccines. All vaccines conferred satisfactory immunity in sheep tested over periods varying from 16 days to 7 months. Culture vaccines were preferable and among them vaccine containing alum was prefered. Thorold (1953) reported that a potent anti-chauvoei vaccine could be produced by using culture from intussuscepted cellophane tubing. Doses of 1.0 ml. and 2.0 ml. in sheep and cattle respectively elicited higher degree of protection against challenge. This vaccine could be stored in wet and dry form for upto one year without apparent loss of antigenicity. Buddle (1954) reported that the formalised alum-precipitated vaccine prepared with an 18 hour old culture of Clm chauvoei in beef broth containing glucose and cystein hydrochloride when tested in sheep was found to be better in potency when compared with the two other Newzealand vaccines and an American vaccine.

In India, Iyer and Ramchandran (1954) reported that there was no difference in the immunising value of the 38-hour old and 14-day old Noguchi Medium vaccine prepared by mixing equal parts of Cl. chauvoei and Cl. septicum. Also there was no significant

difference in the immunising value of vaccines prepared in cysteine broth and Noguchi Medium. Addition of 1% alum as adjuvant to the vaccine appeared to improve the quality of immunity established by both the Cyteine broth and Noguchi Medium vaccines. Bull calves, buffalo calves and guinea pigs protected with this vaccine were able to withstand a challenge dose of 10 M.L.Ds. of virulent Cl. chauvoei culture in most cases.They further stated that higher concentration of the alum-precipitated vaccine by removing the supernate further enhanced the immune response.

Kagan et al. (1961) described the procedure for concentrating Cl. chauvoei by the vat method. The organism was grown in liver medium containing 0.2% glucose at pH 2 - 7.4 for 24- 36 hours. After filtration, the fluid was formalised at 37°C for 3 days then aluminium hydroxide was added and the mixture held for 2-3 days at room temperature. The upper 3 quarters of the mixture was decanted and the remainder was used as a vaccine in 2 ml. doses for calves. They reported that this vaccine was superior to the 5.0 ml. of the ordinary formalised vaccine. Grigoriu et al. (1962) observed that when the supernate of vaccine containing alum hydrogel was discarded, the sediment possessed better immunising properties and therefore they felt that the supernatant did not contain the immunising principle and could be dispensed with without any prejudice to the vaccine. Further, Grigoriu et al. (1965) reported that concentrated vaccine with excess aluminium hydroxide was found in the field trials to confer

stronger immunity in cattle with 5.0 ml. dose than the original broth vaccine in 10 ml. doses. Antibodies were demonstrable after 6 months in the serum of cattle vaccinated with the concentrated vaccine but not in those vaccinated with the other vaccine.

Nilakantan (1965) reported the comparative efficacy of Black quarter vaccine prepared in three different types of media such as (1) Livermeat infusion broth, (2) Livermeat acid digest broth, and (3) Liver papain digest broth. Of the three media studied, livermeat infusion broth was used for major portion of the work. The preparation of this medium was simple and even Cl. chauvoei which is fastidious in its growth requirements grew profusely. It was observed that goat liver and meat were as good as tissues from Ox. The preparation of papain digest broth was also comparatively simple, while that of acid digest broth was rather complicated. Potency test of the vaccine prepared from the various media mentioned above revealed the superiority of liver-meat infusion broth vaccine. However, vaccines prepared from all the 3 media were far better than that prepared from glucose broth containing pieces of fresh rabbit kidney. He observed that neutralization and alum precipitation improved the keeping quality as well as potency of the vaccine.

Tomic et al. (1960) and Ryder (1968) reported that two strains of Cl. chauvoei were cultured in two different medium and 3 vaccines were prepared from each, namely, a buffered formalised whole culture, a formalised culture absorbed with 20%

alhydrogel, and another precipitated with potassium aluminium sulphate. All contained 2 x 10^8 organisms per ml. The vaccines were tested in guinea pigs. The alum-precipitated and the alhydrogel adsorbed vaccines protected 67% of them against challenge as compared to 30% survival with broth vaccine.

IMMUNOGENIC ANTIGEN

1. Haemorrhagic septicaemia :-

Like many other organisms, Past. multocida possesses an array of somatic and envelope antigens, but only a few of them are immunogenic. The vaccine, to be effective, should contain the immunising antigen either in pure isolated form or should possess an adequate amount of this antigen in whole cell culture.

Priestely (1936a, 1936b) and Schuitze (1932) are among the poineers who studied the antigens of Past. multocida type I. Priestely, by the help of agglutination and agglutinin-absorption tests, identified a thermostable somatic and heat labile envelope antigens. Schuitze revealed that unlike Past. pestis, only virulent strains of Past. multocida possessed envelope antigen.

The following antigenic fractions have been isolated from Past. multocida :

Polysaccharides :-

Dingle (1934) obtained serologically active polysaccharide from strains of Past. multocida. Yusef (1935), while working for the serological classification of the strains of Past. multocida, observed serologically specific capsular substances. Carter (1952), by heating the organisms at 56°C, extracted soluble type of specific antigens from the capsules. Carter and Annan

(1953) further analysed these antigens and found them to be polysaccharides. They also observed that the polysaccharides evoked definite immunity in mice. It may have been due to the fact that their polysaccharide had become complexed with or contaminated with nucleic acid, which would be expected from their method of preparation. This is the only ~~six~~ isolated observation suggesting that the capsular polysaccharides can be categorised as immunogens.

Knox and Bain (1960) observed that the polysaccharide was most abundantly recovered from cells grown on solid media, but the quantity was greatly reduced in broth-grown cells. It was intermediate in amount when bacteria were grown in a cellophane suspended in broth. In liquid media, polysaccharides passed readily into solution, leaving the cells agglutinable by specific anti-sera. On solid media, the polysaccharide remained on the surface, rendering the cells relatively in-agglutinable. They further observed that harvested cells of the so-called 'blue' i.e., non-capsulated, variants of Past. multocida type I possessed very little polysaccharide. The purified polysaccharide were haptens and depended for their antigenicity on re-inforcement by protein (Dhanda, 1959a; 1960; Dhande et al., 1959; Knox and Bain, 1960; Carter and Bain, 1960). These observations were confirmed separately by other workers.

concluded that capsular polysaccharide *per se* was a complex hapten. His opinion was based on the findings that the capsular polysaccharide, when used for immunising hill cattle, proved non-immunogenic even in doses upto 9000 micrograms. This polysaccharide, however, when adsorbed on erythrocytes or killed streptococci, produced high titred antipolysaccharide serum antibody, though the anti-sera so produced exhibited no passive mouse protective activity. Even the best preparation of the polysaccharide antigen absorbed very little of the mouse-protective activity from rabbit type I anti-serum. This suggested inferiority in the immunological integrity of polysaccharide antigen.

Bain (1960) on the basis of immuno-fluorescent technique and Dhanda (1960) by agglutination-inhibition test confirmed that capsular polysaccharide was located superficially on the surface of intact cultured cells of *Past. septica*.

Even though the capsular polysaccharides present in *in vitro* grown cells form precipitate, fix complement, and possess some antiphagocytic and virulence enhancing factor in low concentrations, they are absent from or not produced by *in vivo* grown pasteurellas. Therefore, no pathological attribute can be assigned to them. However, they do have their effect upon *in vitro* grown cells against the bactericidal and opsonic action of antibodies by their interference effect (Bain, 1960).

Lipopolysaccharides :-

The idea of obtaining lipopolysaccharide was gathered

from the work of Davies and other workers. Davies (1956) obtained this lipopolysaccharide from Past. pestis, while Machennan and Rondle (1957) extracted pyrogenic non-toxic lipopolysaccharides from acetone-killed cells of Past. multocida type V with 45% phenol on the lines of Westphal et al. (1952). They found them to be responsible for type specificity, which paralleled the immunological specificity established by passive-mouse protection test of Roberts (1947). Machennan and Rondle (1957) inferred that similar substance was present in all types of Past. multocida. Dhanda (1960), and Bain & Knox (1961) further extended this work and showed that lipopolysaccharide could be used for sensitizing erythrocytes to agglutination reaction in the presence of specific anti-serum.

Crude iso-electric precipitates from saline extracts of whole cells as shown by Bain (1955a) was erratically toxic for rabbits. Bain & Knox (1961) reported that it was due to the lipopolysaccharides. Their preparations always killed rabbits in a dose of 500 micro-grams intra-venously, but the effect of lower dose was inconsistent. Therefore, the determination of LD_{50} was virtually impossible with moderate numbers of animals. Some rabbits receiving 20-50 micrograms intravenously died in 3-4 hours; a few of those receiving doses between 50 and 500 micrograms died in form 2 - 36 hours whilst other survived. They also noticed toxic changes in liver cells, kidney tubules and heart muscle. Haemorrhages were seen at times in the adrenals and invariably

in the spleen. They regarded spleenic haemorrhage as an indicator of toxicity due to lipopolysaccharide. Two weeks old calves, after receiving i/v 300 micro-grams and 800 micro-grams respectively of lipopolysaccharide collapsed after two hours. The blood of cattle of Haemorrahagic septicaemia yielded from 20 to 100 microggrams dry weight of pasteurellae per litre. On the basis of assumption that these cells would also contain 0.1% of lipopolysaccharide as contained by in vitro grown cells, the authors concluded that an adult infected ox might have a gramme or more of pasteurellae in its blood and over a milligram of lipopolysaccharide. It was antigenic and produced in rabbits precipitins which was identified by these authors on Ouchterlony plates. By repeated tests, purified lipopolysaccharide in doses of 20 - 200 micro-grams protected on an average 20% of mice against challenge with 1000 ID_{50} bacteria i/p 3 weeks later. All the above preparations removed some but not all of the mouse protective property of rabbit and cattle anti-sera. All mice in a group of six, given 5 mouse protective dose (m.p.d.) of absorbed serum, died on challenge, and those which received 20 mouse protective dose (m.p.d.) survived.

Dhanda (1960) had reported that capsulated bacilli were not agglutinable in sera produced in rabbits against this fraction conjugated with lipoprotein obtained from Shigella dysenteriae, while the non-capsulated bacilli got agglutinated. These findings give enough justification to believe that the

specificity of the somatic antigen is resident in the carbohydrate moiety of the lipopolysaccharide fraction (Dhanda, 1959b), while that of the capsule in the polysaccharide fraction in combination with protein (Dhanda, Negi and Sekariah, 1959). Dhanda on the basis of these experiments thought it plausible to theorise that close parellelism observed between the types established on the basis of precipitation, or r. b. c.-absorbing property of the lipopolysaccharide or capsular polysaccharide and those by passive mouse-protection test in mice is only a manifestation of the activity of these antigenic fractions that perhaps render the induced immunity specific.

Dhanda (1960) found lipopolysaccharide, in contrast to a findings of Bain and Knox (1961), to be a hapten and a good pyrogen for rabbits, resembling in this way lipopolysaccharides obtained from several gram negative bacteria but differing from them in being almost devoid of toxicity, killing mice and rabbits only in doses of 5 - 7 mg. Similar lipopolysaccharides were also isolated by Grard and Gallut (1951) from Past. tularensis and by Davies (1956) from Past. pastis.

Agglutination-inhibitation tests carried out by Dhanda (1960) indicated the lipopolysaccharides to be absent on the capsule and to be predominant in the cellular body of the organism. This conclusion was supported by the findings of Devies (1956), that it was necessary to remove the surface proteins in non-

capsulated cells with 2.5% NaCl before the lipopolysaccharide could be extracted.

Specific lipopolysaccharides of Past. multocida were extracted and the antibody against them was measured by indirect H. A. test. The lipopolysaccharides of type A (Robert type II), Type B (Robberts type I) and type D were extracted from moderate harvests of organisms grown on serum agar by phenol extraction method. The least amounts of the lipopolysaccharides to yield maximal H.A. reaction were in the range of 2 to 10 µg.Ø milli litre of 1% erythrocytes. The lipppolysaccharides were adsorbed equally well to fresh and formalinzed human group 'O' red cells (Carter and Rappy, 1963).

Proteins :-

Two protein antigens have been isolated from Past. multocida type I and have been designated as fractions Ib and II (Bain, 1955a, Dhanda, 1959b, Dhanda, 1960). Similar protein fractions have been obtained by Amies (1951), and Baker et al. (1952) from Past. pestis. Baker also named his fraction as FI_A.

These protein fractions were isolated from water soluble 2.5% saline extracts of Past. multocida type I as crude protein, consisting of several immunologically distinct substances including polysaccharide, lipopolysaccharide, nucleic acid, protective protein and toxic protein.

Fraction IB :-

Dhanda (1960) crystallysed the protein fraction (IB) from crude 2.5% NaCl and 0.5 M potassium thiocynate extracts by fractional precepitation with ammonium sulphate on the lines of Baker et al. (1952).

Of the two protein antigens, FI isolated from water souble saline extracts of Past. septica proved to be non-toxic but possessed high immunogenic value for mice, rabbits, guniea-pigs and hill cattle (Dhanda, 1960). A fraction of this (FIA) was a protein linked with polysaccharide while another fraction (FIB) was a protein but devoid of carbohydrate which he could crystalize as referred to above from $(NH_4)_2SO_4$. The other antigen was found by the author to be proteinaceous in nature, proving lethal for mice and rabbits. It was relatively homogeneous, forming perhaps the soma of the bacilli (Dhanda, 1959a).

From the point of biological significance, the two protein fractions seem to be important, as far as immunization against H. S. Subcutaneous dose of 500 /ug. of fraction IB rendered hill cattle solidly immune (Dhanda, 1960) and buffaloes (Bain, 1955a).

Dhanda et al. (1960) found that anti-fraction IB antibodies became defectable in animals as early as on the 4th day and reached a maximum titre by the 18th day following anti-genic stimulation. The authors found a significant correlation

between the results of anti-fraction IB titre and direct challenge of vaccinated animals.

Fraction II :-

Carter and Bain (1960) mentioned that some of the toxicity of cultures of Past. multocida can be attributed to glycolipid or "bovin antigens" first described by Pirosky (1938a, b, c). Ajl et al. (1955) described a toxin from Past. pestis. Similar toxic protein (FII of Dhanda) has been identified from type B cultures of Past. multocida by Dhanda (1960) and Knox and Bain (1960). The LD_{50} of the toxin for mice was 5 micrograms by I/P route and 2.5 micrograms by i/v route. It was found to get inactivated at the temperature of 56°C or higher within 30 minutes. A potent anti-toxin could be prepared with Freund's adjuvant, which neutralized the toxin. The antigen, as found by Dhanda was homogenous and formed a part of the soma of the bacilli. In the same paper, Dhanda demonstrated the sensitization of tannicacid-treated erythrocytes with purified toxin which provided a useful serological tool for determining anti-toxin levels of sera.

The crude isoelectric fraction of the soluble protein antigens (FII of Dhanda, 1960) of encapsulated cells of Past. septica type I which was extracted with 0.5 M potassium thiocynate on the lines of Amies (1951) was found by Bain (1955a) to be

toxic to rabbits but not to mice in doses of 50 micrograms to 1 milligrams and this result was subsequently confirmed by Dhanda (1960). He explained that this observed low toxicity was due to the well known toxoiding action of potassium thio-cynate, in an anology with similar effect observed in the case of diphtheria toxin (Hosoya et al., 1944, 1953) and in case of staphylococcal toxin (Miyasaik and Hiraidi, 1957).

From the foregoing review, it can be seen that protein fraction of capsular extracts is strongly immunogenic in cattle, buffaloes and mice. The crude protein contains some of the polysaccharides and mucoprotein components as well as the lipopolysaccharide. However, it is extremely difficult to purify this fraction and confine the activity to a single homogeneous antigen.

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2. Black quarter :-

In India, serological study during outbreaks of Black quarter in cattle revealed that most of the cases of Black quarter were due to Cl. chauvoei. Only a few were caused by Cl. septicum either alone or in conjunction with Cl. chauvoei (Iyer, 1952).

Felix and Robertson (1928), in their classical work on the "O" and "H" agglutinins of Cl. septicum, reported considerable overlapping of the "O" antigens. The experiment of Robertson and Felix (1930), however, showed that the "O" antibody was type specific. Later, Henderson (1934) reported sharing of a second "O" antigen in a low titre by all strains of Cl.chauvoei which were responsible for a varying degree of cross-protection against Cl. septicum infection.

On the other hand, Roberts (1931) carried out flagellar and somatic agglutination tests with English strains of Clostridum chauvoei and reported the presence of on a heat stable "O" antigen common to all bovine and ovine strains, and a heat - libile 'H' antigen which was specific for host-specific strains. He suggested that bovine and ovine strains form two distinct "groups" of the bacterial species of Cl. chauvoei. Immune serum derived against bovine strains to conferred protection against infection with the bovine type. Henderson (1932), however, found that all strains of Cl. chauvoei examined, possessed a

common 'H' antigen, except the English ovine strain, which in addition to 'H' component which was also present in the ovine and bovine type.

Roberts (1933) reported that guinea pig could be afforded a moderate degree of immunity against artificial infection with Cl.chauvoei by inoculating a large quantity of 'O' antigen of the organism. However, he failed to confirm that this antigen conferred almost complete immunity against 100 and 1,000 lethal doses of Cl, chauvoei. Thus he doubted the value of the artificially activated spore suspension ('O' antigen) as an agent for the estimation of immunity.

Therefore, he concluded that immunisation of animals by 'O' antigen alone was unlikely to be of practical value.

Moussa (1959) investigated into the antigens of spores and vegetative bacilli of the genus Clostridia. Earlier to this no work seems to have been done on the spore antigens of Cl.septicum and Cl. chauvoei or any other clostridial organisms except Cl. sporogenes by Mandia and Bruner (1951). Though Defalle (1902) demonstrated the antigenicity of the spores as early as 1902, no attempts were made to utilise it as a means of classifying clostridia. Moussa (1959) examined 37 strains of Cl. septicum and 38 of Cl. chauvoei, isolated in Britain, France and U.S.A. and reported that Cl. septicum strains possessed two 'O' and 5 'H' antigens and could be divided into 6 groups. On the other

hand, all but two Cl. chauvoei strains possessed a single 'O' antigen, but could be divided into two groups based on two 'H' antigens which, were distinct from those of Cl. septicum. Further, he stated that all strains of Cl. septicum and Cl. chauvoei ~~x~~ posssessed a single common spore(s) antigen except in two strains of Cl. chauvoei which showed no antigenic relationship to each other, but shared an antigen similar to Cl. septicum. He concluds that the serological and other differences between Cl.septicum and Cl. chauvoei do not justify regarding them as two distinct species, and that they might very well be regarded as type A and B of the single species of clostridium (Mace, 1888; Ford, 1927).

From the above account, it will appear that antigen protective against Black quarter have not been as thoroughly investigated as done for those against H. S. However, it seems clear that whole cell culture of Cl. chauvoei which possesses this antigen, is necessary to for immunisation of cattle against Black quarter.

EVALUATION OF IMMUNITY IN CATTLE

1. Haemorrhagic septicaemia :-

Various laboratory procedures have been described for determining immune status of cattle immunised against Haemorrhagic septicaemia. While actual challenge of immunised animals with virulent homologous organism remains the method of choice, several serological tests have been reported to detect the immune response of immunised hosts. Of these, agglutination test (Bain, 1954d, 1955b), agar agglutination test (Bain, 1955a), complement fixation test (Oster et al., 1952; Bain, 1955b) & mouse protection test (Roberts, 1947; Bain, 1955b) have received considerable attention during the last two decades.

However, it is admitted on all hands that the mouse protection test is the best laboratory procedure to evaluate the immunity of animals vaccinated against H. S. Carter (1955) described haemagglutination test for the serotyping of Past. multocida. Later, Carter and Rappay (1963) suggested that study should be further extended to find out the correlation between the degree of haemagglutination and immunity measured by challange or animal protection test.

2. Black quarter :-

So far no reliable laboratory test has been described

to measure the immunity of animals against Black quarter except exposure of vaccinated animals to artificial infection or passive protection of susceptible hosts against challenge infection. This has been confirmed by several workers (Roberts, 1933; Piercy, 1951; Iyer and Ramchandran, 1954; Tomic et al., 1960; Nilakantan, 1965; Ryder, 1968).

C H A P T E R - III

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Haemorrhagic septicaemia vaccine :-

(i) STRAIN:

A virulent strain of Past. multocida P_{52} was obtained from the Livestock Research Station (L.R.S.), Bihar, Patna.

The strain was passaged periodically through buffalo calves and the isolate was checked for purity by microscopic and cultural examination. It was maintained in phase I in sheep blood agar slant and stored in refrigerator. This was subcultured at fortnight interval in blood agar slant. The stock culture was examined for purity each time before it was used subsequently in the experiment.

(ii) MEDIA:-

As it will appear in the subsequent section, 4 groups of combined H. S. and B. Q. vaccines were prepared and studied for their immunogenicity. For this purpose, Past. multocida strain P_{52} in phase I was grown on liquid as well as in solid media as described below :-

(a) Liquid medium :-

Nutrient broth as used at Livestock Research Station, Bihar, Patna for the preparation of H. S. broth vaccine was

prepared except that beef was substituted with lablemco. The composition of the medium was as follows :-

Peptone (Difco-bacto)	-	1.0%
Sodium chloride.	-	0.5%
Lab-lemco.	-	1.0%
Distilled water.	-	100 ml.

The pH was adjusted to 7.4. The medium was transferred in different volumes in flasks and tubes as required. It was autocalved at 15 lbs. pressure for 1/2 an hour. After that it was incubated for 24 hours and then stored in the refrigerator until use.

(b) Solid medium :-

Yeast extract creatinine-agar medium as described by Dhanda et al. (1956) was prepared except that Bacto-agar powder was used in concentration of 3% instead of agar shreads. The media was prepared as follows :-

Yeast extract powder (Difco-Bacto)	-	0.5 gm.
Peptone (Difco-Bacto)	-	1.0 gm.
Lab Lemco.	-	1.0 gm.
Creatinine.	-	0.1 gm.
Sodium chloride.	-	0.5 gm.
Bacto agar powder.	-	3.0 gm.
Distilled water.	-	100 ml.

The final pH. of the medium was adjusted to 7.2. Lateron, it was transferred into Rouxe flasks which were then autocalved at 15 lbs. per 1/2 an hour. In order to avoid formation of excess water of condensation, the Rouxe flasks were slanted when the temperature of the medium came down to about 50°C. The Rouxeflasks were incubated over night in inverted position for drying and then they were stored in the refrigerator. Prior to inoculation of the culture, they were further incubated for 6 hours.

(iii) VACCINE PRODUCTION:

(a) Haemorrhaegic septicaemia broth vaccine:-

For the preparation of Haemorrhaegic septicaemia broth vaccine, Past. multocida strain P_{52} maintained in sheep blood agar slant was transferred into nutrient broth tube and incubated at 37°C for 18 hours. After that the growth was examined for purity by microscopic examination and sub-culturing on blood agar plate. Two 500 ml. flasks, each containing 250 ml. of nutrient broth medium, were inoculated each with 0.5 ml. of 18 hour old broth culture of Past. multocida. The flasks were incubated for 18 hours and the growth checked for purity. To this was added 1.25 ml. of formaline (0.5%) which was further incubated for 48 hours to ensure killing of the organisms. After 48 hours incubation, the formalised broth culture was tested for sterility. For

this, it was inoculated into tubes of blood agar slant, nutrient broth and Robertson cooked meat medium and incubated for 48 hours. When found sterile, this H. S. broth vaccine was stored in refrigerator for further use.

(b) Formalised agar washed H. S. culture :-

Five Rouxeflasks containing yeast extract-creatinine-agar medium were inoculated with 3.0 ml. of 18 hour old broth culture of Past. multocida. The inculum was evenly spread on ag surface by gentle forward and backward motion. The flasks were then left on the table for 1/2 an hour to allow the organisms to settle nicely on the surface of the medium. They were later inverted and incubated for 18 hours. After incubation, the flasks were examined for growth and purity as described above. Thereafter, the growth from each flask was killed and washed with for 15.0 ml. of 0.5% for mal-saline which was later collected in sterile flask.

The formal-killed agar-washed culture obtained from 5 Rouxeflasks was pooled, which was further diluted with 0.5% formol-saline to obtain an opacity comparable with Brown's opacity tube No. 6. Thereafter, it was incubated for 24 hours to ensure killing of the organisms. The sterility test was made as described earlier. This product was required to be prepared quite frequently for use in different combinations of antisera in B. Q. vaccine. The product was stored in the refrigerator and the pH. was

this, it was inoculated into tubes of blood agar slant, nutrient broth and Robertson cooked meat medium and incubated for 48 hours. When found sterile, this H. S. broth vaccine was stored in refrigerator for further use.

(b) Formalised agar washed H. S. culture :-

Five Rouxeflasks containing yeast extract-creatinine-agar medium were inoculated with 3.0 ml. of 18 hour old broth culture of Past. multocida. The inculum was evenly spread on agar surface by gentle forward and backward motion. The flasks were then left on the table for 1/2 an hour to allow the organisms to settle nicely on the surface of the medium. They were later inverted and incubated for 18 hours. After incubation, the flasks were examined for growth and purity as described above. Thereafter, the growth from each flask was killed and washed with 15.0 ml. of 0.5% for mal-saline which was later collected in a sterile flask.

The formal-killed agar-washed culture obtained from 5 Rouxeflasks was pooled, which was further diluted with 0.5% formol-saline to obtain an opacity comparable with Brown's opacity tube No. 6. Thereafter, it was incubated for 24 hours to ensure killing of the organisms. The sterility test was done as described earlier. This product was required to be prepared quite frequently for use in different combinations of H. S. - B. Q. vaccine. The product was stored in the refrigerator for

further use.

2. Black quarter broth vaccine :

(i) STRAIN :-

A highly virulent strain of Cl. chauvoei '49', used for the production of B. Q. broth vaccine was obtained from the Research Officer (Biological Product), Livestock Research Station, Bihar, Patna for use in the experiment. The strain was received and subsequently maintained in Robertson's Liver meat infusion broth. This was stored in the refrigerator.

(ii) MEDIA :-

Liver -infusion -thioglycollate -broth described by Seetharaman and Sinha (1963) and Nilakantan (1965), and used for the production of B. Q. broth vaccine at Livestock Research Station, Patna was prepared except that beef was substituted with lab-lemco. The composition of the medium was as follows :-

Liver tissue	--	..	250 gm.
Lab-lemco	..	..	10.0 gm.
Peptone (Difco-bacto).		..	10.0 gm.
Sodium chloride	..	..	5.0 gm.
Sodium thioglycollate.		..	1.0 gm.
Distilled water	..	..	1,000 ml.

It was sterilised at 15 lbs. pressure and the pH. was

adjusted to 7.4. After sterility test, it was stored in the refrigerator for use.

For the preparation of broth vaccine, 2 one litre flasks, each containing 500 ml. liver infustion-thioglycollate - broth were warmed up to boiling point in water bath for 5 minutes and then cooled to 45°C. Thereafter, they were inoculated each with 0.5 ml. of 48-hour old broth culture of Cl. chauvoei strain '49'. The inoculum was mixed by shaking and incubated for 72 hours. After 72 hours incubation, flasks were removed from the incubator and the growth examined microscopically by smear examination for purity. Later, 25.0 ml. of 10% formaline solution was added to the each flask (final formaline concentration 0.5%) and shaken well. It was further incubated for 48 hours at 37°C to ensure complete killing of the organisms.

Sterility test of the formalised broth vaccine was done by culturing in blood agar plates, nutrient broth, and Robertson's liver infusion broth. This was further checked by inoculating it into guinea pigs along with 0.2 ml. of 5.0% calcium chloride intra-muscularly. The strile broth vaccine was stored in refrigerator for subsequent use.

Determination of the optimum concentration of aluminium potassium sulphate for the preparation of B.Q. alum-precipitated broth vaccine:

First of all, the optimum concentration of alum to be used for thepreparation of B.Q. alum-precipitated broth vaccine

was determined. A 10% sterile solution of aluminium potassium sulphate ($Al_2 (SO_4)_3 K_2SO_4$, 24 H_2O, mol. wet. 948.81) in distilled water was used for this purpose.

PROCEDURE :

Five milli-litres of formalised B.Q. broth vaccine prepared as described earlier were taken in 7 tubes. To each of them, 10% aluminium potassium sulphate solution was added so as to give final concentrations of 0.2% to 0.8% from 1st to 7th tube respectively as shown in Table I. These tubes, after addition of the alum solution, were left overnight in the incubator. After 24 hours, the tubes were examined for maximum precipatation. As it will appear from the Table I, maximum precipitation occurred in tube no. 5, 6 and 7. Supernatant of the last 3 tubes (No. 5, 6, and 7) showing maximum precipitation was transferred into another set of 3 tubes separately in equal amounts taking care not agitate the precipitate. To all these 3 tubes, alum solution was further added, making a final concentration to 2% in each of them. After incubation, these tubes were examined for the presence of visible precipitate if any. The results are shown in Table II.

It can be seen that further precipitation did not occur in the tubes which were previously treated with the alum concentration of 0.6% and above. Thus, 0.6% solution of aluminium potasium sulphate was considered to be optimum for getting maximum precipitation of B.Q. broth vaccine.

T A B L E - I

Protocol of titration of Aluminium Potasium-sulphate.

Serial number	Quantity of B.Q. broth vaccine used.	Quantity of 10% sterile solution of aluminium potassium sulphate added.	Percentage of aluminium potassium sulphate in the tube.	Degree of precipitation.
1	5.0 ml.	0.1 ml.	0.2%	+
2	5.0 ml.	0.15 ml.	0.3%	+
3	5.0 ml.	0.2 ml.	0.4%	+ +
4	5.0 ml.	0.25 ml.	0.5%	+ + +
5	5.0 ml.	0.3 ml.	0.6%	+ + + +
6	5.0 ml.	0.35 ml.	0.7%	+ + + +
7	5.0 ml.	0.4 ml.	0.8%	+ + + +

TABLE - II

Protocol for testing sediment (precipitable antigen) in the supernatant.

Serial number	Number of the tubes showing maximum precipitation.	Quantity of supernatant	Quantity of 10% aluminium potassium sulphate solution added.	Percentage of aluminium potassium sulphate in the tubes.	Precipitation if any.
1	5	3.0 ml.	0.6 ml.	2%	No precipitation.
2	6	3.0 ml.	0.6 ml.	2%	No precipitation.
3	7	3.0 ml.	0.6 ml.	2%	No precipitation.

However, for the preparation of B. Q. broth alum-precipitated vaccine, 1% instead 0.6% of alum was used in order to correct any experimental error as well as to cover the extra bulk of pasteurella anaculture.

Determination of dose of B. Q. broth alum-precipitated vaccine:

The dose of this vaccine was standardised by comparing the volume of the sediment obtained from formalised B. Q. broth vaccine with that of B. Q. broth alum-precipitated vaccine after centrifugation. For this, 5.0 ml. of B.Q. broth vaccine which constitutes one dose, and 5.0 ml. of B.Q. broth vaccine containing 1.0% of alum solution were taken in graduated sterilised test tubes separately. After overnight standing in the incubator and subsequent centrifugation at 3000 r. p. m. for half an hour, the sediment in the two tubes was measured. After centrifugation, the packed cell antigen of formalised B. Q. broth vaccine represented one vaccinating dose, which ranged between 0.22 to 0.25 ml. On the other hand, the packed cells in alum-precipitated vaccine was 0.5 ml. In order to get 0.25 ml. of packed cells in alum-precipitated broth vaccine, 2 ml. of B. Q. alum-precipitated vaccine was further dealt with similarly which gave the same packed cell volume, i.e. 0.25 ml. after centrifugation as obtained with 5 ml. of formalised B. Q. broth vaccine. This is shown in Table III.

Thus, 2.0 ml. of the B.Q. broth alum-precipitated vaccine constituted one vaccinating dose for cattle equivalent to one vaccinating dose of formalised B. Q. broth vaccine contained in 5 ml. volume.

TABLE - III

Determination of dose of B.Q. broth alum-precipitated vaccine.

Serial number	Quantity of vaccine.		Reading after centrifugation
1	Quantity of B.Q. broth vaccine -	5.0 ml.	0.22 to 0.25 ml.
2	Quantity of B.Q. broth alum-precipitated vaccine -	5.0 ml.	0.5 ml.
3	Quantity of B.Q. broth alum-precipitated vaccine -	2.0 ml.	0.25 ml.

Preparation of different combinations of H.S.- B.Q. vaccine:

Group - 1 : Combined H.S. - B.Q. broth vaccine:

For this, 20 sterile centrifuge tubes were taken. To each tube, 5.0 ml. of formalised B.Q. broth vaccine was transferred with sterile precaution. They were centrifuged at 6,000 r.pm.

for half an hour. Three and half ml. of the supernatant from all the tubes were pipetted off taking care not to agitate the sediment. The remaining amount, i.e. 1.5 ml. liquid along with sediment of all the tubes were pooled in a sterile flask (i.e. total volume 30.0 ml.).

H.S. broth vaccine was also treated in the similar manner and 30.0 ml. of sediment together with supernatant were obtained in a sterile flask. It was added to the pooled B. Q.cell suspension as obtained above and mixed thoroughly.

After sterility test, it was kept in the refrigerator. Thus, 20 doses of combined H.S. - B. Q. broth vaccine were obtained. Three milli-litres of this combined H.S. - B.Q. broth vaccine constituted one vaccinating dose for cattle subcutaneously.

Group - 2 : <u>Combined H.S. broth - B.Q. broth alum-precipitated vaccine</u>:

For the preparation of this vaccine, 40.0 ml. of B.Q. broth alum-precipitated vaccine was taken in a sterile flask with the help of a sterile pipette.

To each of 20 sterile centrifuge tubes, 5.0 ml. of formalised H.S. broth vaccine were taken with the help of sterile graduated pipette and then they were centrifuged at 6,000 r.p.m. for half an hour. Four milli-litres of the supernatant from all

the 20 tubes were pipetted off taking care not to agitate the sediment. The remaining 1.0 ml. liquid along with the sediment from all the 20 tubes were pooled in a sterile flask and the pooled suspension was added to the above flask containing 40.0 ml. of B.Q. broth alum-precipitated vaccine and mixed thoroughly.

After sterility test, it was stored in refrigerator for further use. Three milli-litres of this vaccine constituted one vaccinating dose for cattle subcutaneously.

Group - 3 : Combined H.S. agar-washed-B.Q. broth alum-precipitated vaccine:

For the preparation of this vaccine, 100 ml. of thoroughly shaken B.Q. broth alum-precipitated vaccine prepared as described before was taken in a sterile conical flask with the help of sterile pipette, and then 50 ml. of agar-washed-H.S. formalised culture suspension (matching Brown's opacity tube no. 6) was added to it, and mixed thoroughly. After sterility test, it was stored in the refrigerator for further use. This yielded 50 doses for cattle, 3.0 ml. constituting one vaccinating dose subcutaneously.

Group - 4 : Combined oil-adjuvant H.S. agar-washed-B.Q. broth vaccine:

An eighteen hour growth of Past. multocida on the surface of yeast extract-creatinine agar medium was washed well with

15.0 ml. of 0.5% formol-saline. It was further diluted to match Brown's opacity tube no. 6.

In each 40 sterile centrifuge tubes, 5.0 ml. of B. Q. broth formalised vaccine was poured with the help of sterile pipette and then they were centrifuged at 6,000 r.p.m. for half-an hour. Three millilitres of the supernatant from all the 40 tubes were pipetted off taking care not to agitate the sediment. The remaining 2.0 ml. of liquid along with sediment in each tube were pooled in a sterile flask. This 80.0 ml. of pooled suspension were added to the above 40.0 ml. H.S. agar-washed formalised culture suspension and mixed thoroughly. The procedures for their homogenisation were the same as suggested by Dhanda et al. (1956). First, 10 parts of white oil and 1 part of melted anhydrous lanolin (previously sterilised in autoclave) were emulsified in an electric waring blender to get a stable water-in-oil emulsion. Thereafter, 15 parts of the above combined bacterial suspension was added to the emulsion and was further homogenised so that the droplets of bacterial suspension were distributed throughout the oil. The sterility test was done as described before and then it was stored in the refrigerator. Three millilitres of this vaccine constituted one vaccinating dose for cattle intra-muscularly.

IMMUNISATION OF ANIMALS

Animals :

Only healthy and susceptible buffalo-calves under 1½ and 2 years of age were selected for vaccination. Faecal sample of each experimental buffalo-calf was examined twice for the presence of intestinal parasites. Further, buffaloes calves were checked for serum agglutinin by slide agglutination test against Past. multocida (Dhanda et al., 1959a). Only those which were found to be free from parasites and serum agglutinin were included in this study. A total of 20 buffalo-calves was used for this purpose.

Collection of sera :

Before vaccination, about 10.0 ml. of blood was collected in sterile test tubes from the juglar vein of each animal and the tubes were left in refrigerator overnight in slanted position for separation of serum. The following day, the serum was transferred with sterile pasture pipette into another set of sterile tubes and kept in the refrigerator for further use. This was used for determining normal agglutinin to Past. multocida and normal H.A. titre to be compared with those obtained after vaccination.

Altogether twenty buffalo-calves were used in the experiment. They were divided into 5 groups, each group comprising

4 calves. Soon after collection of blood, each of the four calves in a group was vaccinated with a dose of one of the four groups of combined vaccines, the remaining four calves were left as unvaccinated control. This is presented in Table IV.

T A B L E - IV.

Plan of vaccination with four experimental combined vaccines.

Particulars of vaccines	Buffalo calves nos.	Dose and route of vaccine.
Group - 1.		
Combined H.S.-B.Q. broth vaccine.	1(1),1(2),1(3), 1(4).	3.0 ml. S/c.
Group - 2.		
Combined H.S. broth-B.Q. broth alum-precipitated vaccine.	2(1),2(2),2(3), 2(4).	3.0 ml. S/c.
Group - 3.		
Combined H.S. agar-washed-B. Q. broth alum-precipitated vaccine.	3(1),3(2),3(3), 3(4).	3.0 ml. S/c.
Group - 4.		
Combined oil-adjuvant H.S. agar-washed- B.Q. broth vaccine.	4(1),4(2),4(3), 4(4).	3.0 ml. i/m.
Un-vaccinated control -	C 1, C 2, C 3, C 4.	

Post-vaccination care :

Each group of vaccinated calves was kept in separate room. Body temperature was recorded for 3 days before vaccination and upto 7 days post-vaccination. The presence or absence of s swelling at the site of vaccination was noted for a period of 10 days. Besides, other post-vaccinal clinical syndromes, such as anorexia, depression, lameness etc. were also recorded.

Challenge of vaccinated buffalo-calves with virulent homologous organisms:

Collection of serum :

Immediately before challenge infection, the animals were bled and serum collected in sterile test tubes as described before. The sera were used for subsequent serological tests to be described later.

Procedures for challenge infection:

Past. multocida strain P_{52} was grown in nutrient broth aerobically at 37°C for 18 hours. This culture was further diluted to 10^{-2} with sterile nutrient broth. One ml. of 10^{-2} dilution of this culture constituted the challenge dose to be used subqutaneously.

Similarly, Cl. chauvoei strain 49 was grown in Robertson's liver-meat infusion broth for 18 hours. For challenge

infection, 1.0 ml. of this culture was mixed with 5.0 ml. of 5% calcium chloride solution to be given intra-muscularly to each calf.

Two buffalo-calves of each of the four vaccine groups including the control, as shown in Table IV, were challenged each with Past. multocida and Cl. chauvoei respectively in the doses stated above. The exact time of challenge exposure was noted. All the calves were observed for a period of 10 days.

After challenge, temperature of all the calves were recorded both morning and evening. The exact time of death of these calves was noted. After death, smear made from ear vein and swollen gluteal muscles as was deemed appropriate were examined and post-mortem examination conducted. Suitable cultural examination were made from the heart blood, liver, spleen and the affected gluteal muscles for the isolation of Past. multocida and Cl.chauvoei. The identification and pathogenicity of these isolates were made by biochemical and pathogenicity tests in rabbits and guinea pigs.

Serological evaluation of immunity in buffalo-calves vaccinated against H. S.:

The immune status of calves vaccinated against H. S. was also determined by serological procedures, viz. mouse protection test and indirect haemagglutination test. For this purpose, serum samples of vaccinated calves collected just prior to challenge infection were used. The object of these tests was to determine

the correlation between the result of challenge and serological tests.

Mouse protection test as described by Bain (1955b) and indirect haemagglutination test of Carter (1955) were followed. The later was performed on perspex H.A. plate using 1.0% antigen-sensitized human 'O' erythrocytes obtained from P. W. Medical College Hospital, Patna. The H. A. titres were finally recorded after overnight refrigeration of perspex plate.

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C H A P T E R - IV

R E S U L T S

R E S U L T S

POST-VACCINAL REACTION:

As shown in Table IV, 16 buffalo-calves, divided into four groups, were vaccinated with 4 groups of combined H.S.-B.Q. vaccines. Four un-vaccinated buffalo-calves acted as control.

Clinical :

None of the calves vaccinated subcutaneously in the left side of neck with group 1 vaccine (combined H.S.-B.Q.broth vaccine) showed local swelling at the site of inoculation. On the other hand, all the calves vaccinated with vaccine groups 2 and 3 (combined H.S. broth- B.Q. broth alum-precipitated vaccine and combined H.S. agar-washed - B.Q. broth alum-precipitated vaccine respectively) showed diffuse, hot and painful-swelling about 1 to 1.5 inches in diameter. However, the swelling subsided spontaneously within 2 to 3 days after vaccination. All the 4 calves belonging to vaccine group 4 (combined oils adjuvant-H.S. agar-washed- B.Q. broth vaccine) showed hot, painful and nodular swelling about 3/4"-1" in diameter which persisted for 4 to 5 days. After this period, the swelling resolved spontaneously.

In all the 16 vaccinated buffalo-calves, there was rise of temperature, 1° to $4.2^{\circ}F$ above the normal ($100^{\circ} \pm 1^{\circ}F$).The

thermal response was mild in calves of groups 1 to 3, but it was more marked in calves of group 4. In the former, the temperature subsided within 2 to 3 days after vaccination. However, calves in group 4 continued to record higher temperature for 3 to 4 days after which it subsided spontaneously. The clinical response in buffalo-calves following experimental vaccination is presented in Table V.

T A B L E - V

Clinical response in buffalo-calves following vaccination.

Vaccine group	Buffalo-calves numbers	Local swelling		Thermal response	
		Size	Duration	Rise above normal.	Duration.
1	1(1)	-	-	1°F	1 day
	1(2)	-	-	-	-
	1(3)	-	-	1°F	2 days
	1(4)	-	-	1°F	2 days
2	2(1)	1.5"	3 days	2.4°F	2 days
	2(2)	1.0"	2 days	1.4°F	1 day
	2(3)	1.0"	2 days	1.6°F	2 days
	2(4)	1.0"	2 days	2.0°F	2 days
3	3(1)	1.5"	3 days	4°F	3 days
	3(2)	1.5"	3 days	3°F	3 days
	3(3)	1.5"	3 days	4°F	3 days
	3(4)	1.5"	3 days	4°F	3 days
4	4(1)	1"	4 days	2°F	4 days
	4(2)	1"	5 days	4.0°F	3 days
	4(3)	1"	5 days	3.8°F	4 days
	4(4)	3/4"	4 days	4.2°F	4 days
Control (un-vaccinated).	C 1	-	-	-	-
	C 2	-	-	-	-
	C 3	-	-	-	-
	C 4	-	-	-	-

RESULT OF CHALLENGE INFECTION:

Just before challenge infection, the animals were bled and serum collected in sterile test tubes as described earlier.

At the time of challenge infection, each group of 4 vaccinated buffalo-calves (Table IV) were further subdivided into two sub-groups. One sub-group of 2 calves was used for challenge infection with Past. multocida and the other with Cl. chauvoei. Tables VIA and VIB show the results of challenge test.

Result of challenge test for H.S. :

Both the controls buffalo-calves (C 1, C 2) showed slight rise of temperature (102.4°F and 102°F respectively) within 16 hours after challenge infection. By the 24th hour, both recorded high temperature (108°F and 107.6°F respectively). By this time, they showed clinical signs of acute illness, such as accelerated respiration, extreme depression, lachrymation and frothy exudate from mouth. Oedematous swelling developed in the lower region of neck which extended upto brisket. Fever persisted upto the time of death. They died at 38 and 32 hours respectively after challenge exposure.

T A B L E - VIA

Results of challenge test for Haemorrhagic septicaemia.

Vaccine groups	Buffalo-calves Numbers	Interval between vaccination and challenge (days)	Dose of challange*	Buffalo-calves Numbers died.	Interval between challenge and death.	Remarks.
1.Combined H.S.-B.Q. broth vaccine.	1(1) 1(2)	31	1.0 ml. S/c.	1(1)	28 hours.	Past.multocida was isolated from dead calf.
2.Combined H.S.broth-B.Q.broth alum-precipitated vaccine.	2(1) 2(2)	26	1.0 ml. S/c.	2(2)	42 hours.	-do-
3.Combined H.S.agar-washed-B.Q. broth alum-precipitated vaccine.	3(1) 3(2)	22	1.0 ml. S/c.	0	-	-
4.Combined oil adjuvant-H.S. agar-washed-B.Q. broth vaccine.	4(1) 4(2)	20	1.0 ml. S/c.	4(2)	48 hours.	Past.multocida was isolated from dead calf.
Control(unvaccinated)	C 1 C 2	-	1.0 ml. S/c.	C 1 C 2	38 hours.	-do-

*10^{-2} dilution of 18 hour old broth culture of Past.multocida P_{52}.

T A B L E - VIB

Results of challenge test for Black quarter.

Vaccine groups	Buffalo-calves Numbers.	Interval between vaccination and challenge (days)	Dose of challange*	Buffalo-calves Numbers died.	Interval between challenge and death.	Remarks.
1.Combined H.S.-B.Q. broth vaccine.	1(3) 1(4)	31	1.0 ml. i/m	0	-	-
2.Combined H.S.broth-B.Q. broth alum-precipitated vaccine.	2(3) 2(4)	26	1.0 ml. i/m	0	-	-
3.Combined H.S. agar-washed-B.Q. broth alum-precipitated vaccine.	3(3) 3(4)	22	1.0 ml. i/m	0	-	-
4.Combined oil adjuvant-H.S. agar-washed-B.Q. broth vaccine.	4(3) 4(4)	20	1.0 ml. i/m	0	-	-
Control (unvaccinated).	C 3	-	1.0 ml. i/m	C 3	72 hours	Cl.chauvoei was isolated from dead calves.
	C 4	-	1.0 ml. i/m	C 4	72 hours	

* 1.0 ml. of 18 hour old liver meat infusion broth culture of Cl. chauvoei 49 plus 5.0 ml. of 5% Calcium chloride.

Among the experimental groups, one calf each of group 1(1(1)) and group 2(2(2)) died at 28 and 42 hours post-infection respectively. Furthermore, one calf of group 4(4(2)) also died at 42 hour after infection. They registered rise of temperature upto 105°F. None of the calves of group 3 died after challenge infection. They showed only slight rise of evening temperature (103.6°F) after 24 hours which subsided spontaneously the following day.

Result of challenge test for B.Q. :

Both the unvaccinated control calves (C 3,C 4) died at 72 hours after challenge infection. They showed high rise of temperature (106°F-107°F) after 24 hours. There was hot, painful and crepitating swelling at the site of infection at 36 hours after which it became very well pronounced and extended upto both thighs. The skin in the region tended to slogh off. None of the vaccinated groups died as a result of challenge nor did they record any rise of temperature.

MOUSE PROTECTION TEST FOR H.S.:

This test was carried out on a limited scale for the detection of protective antibodies in the sera of buffalo-calves vaccinated with 4 different groups of combined H.S. -B.Q. vaccines. Serum sample of one vaccinated calf each of the 4 vaccine groups (1(1), 2(2), 3(1), 4(2))was selected for this test. The results are shown in Table VII.

TABLE - VII.

Results of Mouse protection test for H.S.

Number of serum samples	Total Number of mice serumised.	Dose of serum S/c.	Dose of challenge infection*	Number died**	Average interval between serumisation and challenge (death).	Remarks.
1(1)	6	0.5 ml.	0.1 ml.	2/6	72 hours	Past.multocida was isolated from dead mice.
2(2)	6	0.5 ml.	0.1 ml.	1/6	105 hours	-do-
3(1)	6	0.5 ml.	0.1 ml.	0/6	-	-
4(2)	6	0.5 ml.	0.1 ml.	1/6	120 hours	Past.multocida was isolated from dead mice.
Controls.	6	Un-serumised.	0.1 ml.	6/6	65 hours	-do-

* 10^{-6} dilution of a 18 hour old broth culture of Past. multocida intraperitoneally.

** Numerator indicates number of mice died, denominator indicates number of mice put to test.

It can be seen that all the un-serumised control mice died after challenge infection while all the 6 mice of vaccine group 3 survived. On the other hand 2 out of 6 mice of group 1 and 1 each of group 2 and 4 died.

INDIRECT HAEMAGGLUTINATION TEST:

This test was performed on the serum samples of all the 16 vaccinated calves including the unvaccinated control. The procedures for the test described by Carter (1955) were followed. The results are presented in Table VIII. From the results it will be clear that H. A. titres of serum samples of 20 buffalo-calves obtained pre-vaccination ranged between 1 : 20 to 1 : 80.

Among the vaccinated groups, 10 out of 16 calves showed H. A. titre of 1 : 320. The H. A. titre in 4 calves was 1 : 160 and in 2 calves 1: 640. It was interesting to find that higher titres were found in 2 calves in vaccine group 3 which survived challenge infection with Past. multocida and gave very satisfactory results on mouse protection test. The H. A. titre of 8 calves challenged with Past. multocida and their correlation with results of mouse protection test are shown separately in Table IX.

T A B L E - VIII

Haemagglutination titres pre-and post-vaccination with combined H.S.-B.Q.vaccines.

Number of serum samples	Reciprocals of serum dilution	
	Before vaccination	Post-vaccination
1(1)	40	320
1(2)	80	320
1(3)	40	160
1(4)	40	160
2(1)	40	320
2(2)	80	320
2(3)	80	320
2(4)	80	160
3(1)	40	640
3(2)	40	640
3(3)	80	320
3(4)	40	320
4(1)	40	320
4(2)	80	320
4(3)	40	160
4(4)	80	320
C 1	40	-
C 2	40	-
C 3	20	-
C 4	20	-

T A B L E - IX

Correlation between the results of challenge, mouse protection and indirect H.A. test for H.S.

Buffalo calf Numbers	Challenge test	Mouse protection test	H. A. titre Reciprocals of serum dilution
1(1)	Died	2/6	320
1(2)	Survived	Not done	320
2(1)	Survived	Not done	320
2(2)	Died	1/6	320
3(1)	Survived	0/6	640
3(2)	Survived	Not done	640
4(1)	Survived	Not done	320
4(2)	Died	1/6	320

Numerator indicates number of mice died, denominator indicates number of mise put to test.

C H A P T E R - V

DISCUSSION

DISCUSSION

A satisfactory vaccine is the one which confers solid immunity of longer duration without producing seveare post-vaccinal reaction. It should be cheap, easy to prepared and administer to the susceptible hosts.

In the present study, post-vaccinal reactions were not observed with the combined H.S. - B.Q. vaccine (Group I). On the other hand, the remaining 3 groups of combined adjuvant vaccines produced local as well as thermal reaction. The oil adjuvant vaccine (Group 4) elicited more pronounced local as well as thermal reactions than the those containing alum-adjuvant (Groups 2 and 3). Such observations attendant with adjuvant vaccines have been frequently reported by workers in the past (Iyer and Ramchandran, 1954, Dhanda et al., 1956, Bain, 1961, Rao and Shanmugham, 1964, Mohan and Nadis, 1966). The reaction, if not unsightly and inconvenient to the host, has been held to be a good index of immunogenicity of the vaccine.

The immunogenicity of the 4 groups of combined vaccines was evaluated by 3 different methods, i.e., challenge test in vaccinated buffalo-calves, mouse protection test and indirect haemagglutination test. The first two methods have been reported to be quite reliable for determining the immune status of vaccinated animals while the haemagglutination test has not yet received

wide application for this purpose.

The result of challenge test was quite discernible within 24 hours. The local hot, diffuse and painful swelling at the site of infection predicted the fate of the calves that lateron succumbed to challenge. These reactions were quite in conformity with those of Bain (1955b).

On scrutiny of the results presented in Table VIA and VIB, it will be clear that of all the 4 experimental vaccines, the vaccine group 3 (combined H.S. agar-washed - B. Q. broth alum-precipitated vaccine) gave most satisfactory result since this vaccine protected both the vaccinated calves against challenge with a fatal dose of a homologous strain of Past. multocida. In all the remaining 3 vaccine groups, the survival rate among calves following a similar challenge infection was 50 percent (Table VIA).

In the present investigation, a quantitative evaluation of the immunising property of sera from vaccinated calves as well as the duration of immunity conferred could not be made. However, several reports have appeared in the literature on the superiority of single oil adjuvant H.S. vaccine over single alum-precipitated or commercial broth vaccine. The time of onset of immunity following vaccination with alum-precipitated and oil-adjuvant vaccines have been reported to be 5 days and 7 days

respectively (Iyer and Rao, 1959). Similarly, the duration of immunity with these vaccines have been found to be 6 months, and one year respectively (Nikiphorva, 1953, Dhanda et al., 1956, Iyer and Rao, 1959). On the other hand, Mohan and Hadis (1966), working with combined H.S. - B. Q. vaccine, reported that the combined oil-adjuvant vaccine was not superior to combined alum-precipitated vaccine when tested in mice. However, in the present study, the challenge test has also revealed the advantage of combined alum-precipitated vaccine (group 3) over oil-adjuvant vaccine (group 4). However, the discrepancy between the survival rate (100%) obtained in the present study and those (33%) of Mohan and Hadis (1966) may be ascribed to the fact that the number of pasteurella organisms contained in one vaccinating dose used by the latter was considerably less (B.W. opacity 2) in comparision to that employed in the present experiment (B.W. opacity 6). It is likely that the combined oil-adjuvant vaccine (group 4) may prove to be superior to the combined alum-precipitated vaccine (group 3) with respect to conferring early and more lasting immunity following vaccination. Further work is necessary to determine the duration of immunity produced by these two vaccines.

In the present challenge test, results obtained with the vaccines group 1 and 2 were apperantely similar to those with the vaccine group 4. Unfortunately, there seems to be no report

in the literature about the comparative efficacy of combined H. S. - B. Q. broth vaccine and combined H. S. broth- B.Q. broth alum-precipitated vaccine. It has been generally held that the commercial H.S. broth vaccine is inferior to the alum-precipitated and H.S. oil-adjuvant vaccines (Bain, 1954b, Dhanda et al., 1956, Iyer and Rao, 1959). Further, agar-washed H.S. vaccine, presently used in the vaccine group 3, has been reported to possess better immunising property than the commercial broth vaccine (Rau and Govil, 1950). Despite the comparable survival rates of calves obtained with the vaccine group 1 and 2 on the one hand and combined oil-adjuvant vaccine on the other, it is reasonable to belive that the protective value (with particular reference to the duration of immunity) of the first two groups can not be compared with the combined oil adjuvant vaccine. This is also attested by longer survival time of the calves of group 4 (48 hours) when compared to those of group 1 and 2 (28 and 42 hours respectively).

The results set out in Table VIB clearly indicate that all the 4 groups of combined vaccines produced solid and dependable immunity. All the calves challenged with a fatel dose of Cl. chauvoei survived whereas, both the controls died of Black quarter. This is quite understandeble from our own experience with the production of B. Q. vaccine which produces solid immunity not deterred by challenge.

The mouse protection test for H. S. was done with limited number of serum samples due to want of adequate supply of mice at the time of experiment. The results are shown in Table VII. It was interesting to find that all the six mice of the vaccine group 3 survived the challenge dose while the death rates in the vaccine groups 1, 2 and 4 were 33%, 16% and 16% respectively. This again emphasises the advantage of vaccine group 3 (combined H.S. agar-washed-B.Q. broth alum-precipitated vaccine) over the other 3 groups. This is in accord with the result of Mohan and Hadis (1966). Further, the survival time of mice with combined oil adjuvant vaccine (group 4) was more (120 hours) than those with group 1 and 2 (72 hours and 105 hours respectively). This suggests the comparative superiority of the combined oil adjuvant vaccine over the combined broth vaccine and combined broth alum-precipitated vaccine (group 1 and 2).

The mouse protection test has been credited to be the best for assessing immunity in vaccinated animals as well as in those with acquired immunity. Bain (1961) considered that survival of even 1 out of 8 mice was indicative of immunity in test animals.

Therefore, the present investigation clearly indicated that while all the four groups of combined vaccines were immunogenic, the combined H.S. agar-washed-B.Q. broth alum-precipitated

vaccine (group 3) was superior to others.

Carter (1955) described haemagglutination test for serological classification of Past. multocida. This test is now accepted as one of the important serological procedures for serotyping of this organism. Carter and Rappay (1963) further reported the use of lipopalysaccharide antigen of Past. multocida in the indirect haemagglutination test and suggested further scope of study to determine the correlation between the results obtained on challenge, haemagglutination and animal protection tests.

From the Table VIII, it will appear that the H. A. titres of buffalo-calves pre-vaccination ranged between 1 : 20 to 1 : 80 while the titres post-vaccination were between 1 : 160 to 1 : 640. This demonstrated an 8-fold increase in the H. A. titre following vaccination.

A comparative study of results obtained on challenge, mouse protection and H.A. tests (Table IX) revealed interesting results. It can be seen that all the calves and mice of the vaccine group 3 survived the challenge infection. The H.A. titre of both the serum samples of this group, i.e., 3(1) and 3(2) was the highest i.e., 1 : 640. This tends to point a fairly close correlation between challenge and mouse protection tests and the H.A. test. Further, serum samples showing titres below 1 : 640 showed variable results inrespect to the survival rate of test animals

used in challenge and mouse protection tests. For instance, the calves showing H.A. titre of 1 : 320 either survived or succumbed to challenge infection. Thus, it can be concluded that the H.A. titre of 1 : 640 was an index of absulute immunity in animals following vaccination.

As pointed out in earlier section, no serious effort has yet been made to evolve a reliable combined vaccine against H. S. and B. Q. The present study has shown encouraging results particularly with resference to the vaccine group 3 (combined H.S. agar-wahsed-B.Q. broth alum-precipitated vaccine). However, the time for onset of immunity and the duration of immunity conferred in vaccinated calves remain to be investigated further. It is necessary that work on this line is extended to enquire into some of these problems associated with combined vaccines. Further, it may be profitable to employ some other adjuvants for the preparation of combined H.A. - B. Q. vaccine. A cheap, satisfactory and dependable vaccine, when evolved on these lines, will go a long way to control the two most fatal scourages of cattle in the country.

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C H A P T E R - VI

S U M M A R Y

SUMMARY

In the present study, an attempt was made to prepare a number of combined vaccines with and without adjuvant against Haemorrhagic septicaemia and Black quarter in cattle. The following vaccines were tried :-

Group - 1 : Combined H.S. broth-B.Q. broth vaccine.

Group - 2 : Combined H.S. broth-B.Q. borth alum-precipitated vaccine.

Group - 3 : Combined H.S. agar-washed-B.Q. broth alum-precipitated vaccine.

Group - 4: Combined oil adjuvant H.S. agar-washed-B.Q. broth vaccine.

The immunising property of these groups of combined vaccines were tested in susceptible buffalo-calves by challenge test, mouse protection and indirect haemagglutination tests.

Buffalo-calves immunised with 3.0 ml. each of the above four vaccines were subsequently challenged with a fatal dose of homologous virulent strains of Past. multocida P_{52} and Cl. chauvoei 49. The challenge test revealed advantages of combined H.S. agar-washed-B.Q. broth alum-precipitated vaccine (100% survival rate) over the remaining 3 groups of vaccines (50% survival rate). The latter demonstrated immunising property of

equal value.

The mouse protection test strongly indicated the immunogenicity of all the 4 groups of vaccine. This test unequivocally confirmed the immune status of test animals following vaccination. The results, which were in conformity with challenged test, further demonstrated the superiority of vaccine group 3 (combined H.S. agar-washed-B.Q. broth alum-precipitated vaccine) to others. As evidenced by prolonged survival time of mice, the combined oil adjuvant vaccine (group 4) was found to be superior to the combined broth vaccine (group 1) and combined H. S. broth-B.Q. broth alum-precipitated vaccine (group 2).

The indirect haemagglutination test demonstrated an 8-fold increase in H.A. titre following vaccination when compared with the unvaccinated controls. The H.A. titre prior to vaccination ranged between 1 : 20 and 1 : 80 whereas in vaccinated animals, the titres rose between 1 : 160 and 1 : 640. It was interesting to find that the titres were the highest in sera of calves of vaccine group 3 which had protected all the buffalo-calves and mice on challenge and mouse protection tests.

The haemagglutination test further indicated that a titre of 1 : 640 and above was a good index of immunity in vaccinated calves. A close correlation was found between the results obtained on challenge, mouse protection and haemagglutination

tests.

Further studies are suggested to test the onset and duration of immunity in vaccinated calves and to improve upon the immunogenic activity of combined vaccines with other adjuvants. This, when achieved, will go a long way to protect the susceptible cattle population from these fatal enzootics in the country.

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C H A P T E R - VII

BIBLIOGRAPHY

BIBLIOGRAPHY

Ajl, S.J., Reedu-l, J.S., Durrum, E.L., and Warren, J.	1955	J. Bact., 70, 158.
Amies, C.R.	1951	Brit. J. Exp. Path., 32, 259.
Amitrov, V.K., Nechaev, S.P., Medvedev, N.A., and Kazakov, M.Y.	1961	Veterinariya, Moscow, 3, 30. (Vet. Bull., 31, 2824.)
Anon.	1953	J. Amer. Vet. Med. Ass., 123, 242.
Arloing, S., Cornevin, C., and Thomas, O.	1887	J. Inf. Dis., 27, 385 Cited by Stableforth and Galloway, (1959.).
Bain, R.V.S.	1952	Cited by Bain and Jones (1955).
Bain, R.V.S.	1954a	Bull. Off. int.Epiz., 42, 256.
Bain, R.V.S.	1954b	Nature, Lond., 173, 584
Bain, R.V.S.	1954c	Brit. Vet. J., 100, 481.
Bain, R.V.S.	1954d	Ibid., 110, 519.
Bain, R.V.S.	1955a	Ibid., 111, 492.
Bain, R.V.S.	1955b	Ibid., 111, 511.
Bain, R.V.S.	1956	Ibid., 112, 115.

Bain, R.V.S.	1957	Ceylone Vet. J., 5, 2.
Bain, R.V.S.	1960	Nature, Lond., 186, 734.
Bain, R.V.S.	1961	F.S. of Cattle and Buffaloes, South East Asia Treaty Organisation, Bangkok, Thialand.
Bain, R.V.S., and Jones, R.F.	1955	Brit. Vet. J., 111, 30.
Bain, R.V.S., and Jones,R.F.	1958	Ibid., 114 , 215.
Bain, R.V.S., and Knox, K.	1961	Immunology, 4(2) , 122.
Baker, E.E., Sommer, H., Foster, L.E., Meyer, E., and Meyer, K.F.	1952	J. Immunol., 68 , 131.
Basset, J.	1935	Rev. Vet. Toulonse, 87 , 137. (Vet. Bull., 5 , 692.)
Bollinger and Fesser.	1876, 1878	Cited by Stableforth and Galloway, (1959).
Buddle, M.B.	1954	N.Z.J. Sci. Tech. Sect. A., 35 , 395. (Vet. Bull., 25 , 1925.)
Carter, G.R.	1950	Amer. J. Vet. Res., 11 , 252.
Carter, G.R.	1951	Ibid., 12 , 326.
Carter, G.R.	1952	Canad. J. Med. Sci., 30 , 48.

Carter, G.R.	1955	Amer. J. Vet. Res., 16, 481.
Carter, G.R., and Annan, E.	1953	Amer. J. Vet. Res., 14, 475.
Carter, G.R., and Bain, R.V.S.	1960	Vet. Rev. Annot., 6, 105. (Vet. Bull., 31, 35.)
Carter, G.R., and McShery, B.J.	1955	Canad. J. Comp. Med., 19, 177
Carter, G.R., and Rappay, D.E.	1963	Brit.Vet. J., 119, 73.
Curasson, G., and Didier, L.	1932	Rec.Med.Vet.exot., 5, 121. (Vet. Bull., 3, 407.)
Davies, D.A.L.	1956	Biochem. J., 63, 105.
Defalle, W.G-	1902	Ann. Inst.Pasteur., 16, 756. Cited by Moussa (1959).
Dhanda, M.R.	1954	Cited by Dhanda (1958).
Dhanda, M.R.	1958	The Ind.Veterinarian, 1, 13.
Dhanda, M.R.	1959a	Working pper No.8, Meeting on R.S. Manila (Philipines), 30 Nov. 5th Dec. (1959). Cited by Srivastava (1962).
Dhanda, M.R.	1959b	Ind. Vet.J., 36, 6.
Dhanda, M.R.	1960	Ind. J. Vet.Sci. and Anml. Husb., 30, 114.

Dhanda, M.R., Lall, J.M., Das, M.S., and Seth, R.N.	1956	Ind.J.Vet.Sci. and Anml. Husb., 26, 273.
Dhanda, M.R., Lall, J.M., Seth, R.N.	1958	Ind.J.Vet.Sci. and Anml. Husb., 28(111), 139.
Dhanda, M.R., Lall, J.M., Seth, R.N., and Sekariah, P.C.	1959a	Ibid., 29, 30.
Dhanda, M.R., Negi, S.S.,&Sekariah, P.C.	1959b	Golden Jubilee Symposia, Indian Institute of Science, 5. Cited by Dhanda (1960).
Dhanda, M.R., Sekariah, P.C., Lall, J.M.,and Prem Prakash.	1960	Ind. Vet.J., 37, 597.
Dingle, J.H.	1934	Amer. J. Hyg., 20, 148.
Doyle.	1923	Cited by Shirlaw (1957). Brit.Vet.J., 113, 35.
Felix, A., and Robertson, Muriel.	(1928	Brit.J.Exp. Path., 9, 6.
Ford.	(1927	Cited by Moussa (1959).
Gaiger, S.H.	1924	J.Comp. Path., 37, 163.
Geneidy, A.A., Lotfy, O., and El-Affenby, A.M.	1967	Arab.Vet.Med.Ass., 27, 121. (Vet. Bull., 39, 22.)
Grard and Gallut.	(1951)	Cited by Davies (1956).

Grigoriu, N., Carp, N., Jitaru, N., Petrovszky, M., and Paduraru, V.	1962	Lucer.Inst.Pasteur. Buchresti, 6, 289. (Vet. Bull., 32, 4083.)
Grigoriu, N., Carp, N., Petrovszky, M., Butura, I., and Balaci, M.	1965	Lucer.Inst.Corc.Vet.Bioprep. Pasteur, 2 N2, 167. (Vet. Bull., 37, 60.)
Hadley, F. B.	1913	J.Amer.Vet.Med.Ass., 65, 442.
Haddow, J.R.	1933	Annu.Rep.Indian Vet.Res. Inst. Cited by Nilakantan (1965).
Hallesness, P.	1936	Skand.Vet.Tidskr, 26, 225. (Vet. Bull., 7, 7.)
Haq, M.M., and Quader, M.A.	1954	Proc.VIth Pakistan,Sci.Congr. Karanchi, 111, 223. (Vet. Bull., 26, 22.)
Haq, M.M., and Quader, M.A.	1957	Proc.IX Pakistan Sci. Conference, Peshwar, 111, 130. (Vet.Bull., 29, 1684.)
Hardenbergh, J.B., and Boerner, F.	1916	J.Amer.Vet.Med.Assoc., 49, 55.
Hardenbergh, J.B., and Boerner, F.	1917	Ibid., 50, 868.
Hardenbergh, J.B., and Boerner, F.	1918	Ibid., 53, 482.
Heller, H.H.	1920	J.Insf.Dis., 27, 385.
Henderson, D.W.	1932	Brit. J.Exp. Path., 11, 14

Henderson, D.W.	1934	Ibid., 15, 166.
Hosoya, et al.	1944 & 1953	Cited by Srivastava (1962).
Hudson, J.R.	1954	Bull. Off.int.Epiz, 42, 267.
Hyder, G.	1968	Pakist. J.Vet.Sci., 2, 90. (Vet.Bull., 39, 4119.)
Isanko, K.P., and Vasilchenko, I.V.	1937	Trud.Vsezoyuz. Inst.eksp. Vet., 14, 132. (Vet. Bull., 18, 107.)
Iyer, S.V.	1952	Ind. Vet.J., 29, 29.
Iyer, S.V., and Ramchandran, S.	1954	Ibid, 30, 112.
Iyer, S.V., Gopalakrishnan, K.S., and Ramani, K.S.	1955	Ibid., 31, 379.
Iyer, S.V. and Rao, R.D.V.	1959	Ibid., 36, 415.
Kagan, F.I., Nikiforva, N.M. and Kolosova, A.I.	1959	Inst.Vet. preparatov, 8, 124. Cited by Mohan and Nadish (1966).
Kagan, F.I., Kolesova, A.I., Korotaeva, A., Bobrov, P.F., Dvurectenskii, K.A. and Okuneva, V.V.	1961	Trudy nauctno-kontrol Inst.Vet. preparatov, 9, 190. (Vet.Bull, 32, 1041)
Kagan, F.I., Nikiforba, N.M. and Kolesova, A.I.	1961	Veterinariya, Mascow, 8, 21 (Vet.Bull., 32, 1129.)

Karman, P.	1927	Z.Infektionskr Roustiere, 31,266. Cited by Iyer and Ramchandran (1954).
Kausal, R.L.,and Kangude, G.M.	1957.	J.Vet.Res. Mhow, 2, 1.
Kaweh, N., Sohrab, V, and Behar-Sefat, M.	1960	Bull. Off. int.Epiz., 53, 196
Knox, K., and Bain, R.V.S.	1960	Immunology, 3(4), 352.
Kovalenko, Y.R.	1956	Inst.Po. Vet.Preparatev, 6 ,197. (Vet.Bull, 28, 1378.)
LechlaincDe, E., and Valle, E.	1925	Rev.Gen.Med.,34, 293.
LeRoux, G., Gillion, A.	1939	Rec.Med.Vet.Exot., 12, 65. Cited by Shrivastava (1962).
Lignieres (Quoted by Friedberger and Frohner)	1910	Cited by Stableforth and Galloway, (1959).
Mace	1888	Cited by Moussa (1959).
Mason, J.H.	1934	Onderstepoort Journal II, 29. Cited by Iyer and Ramchandran (1954).
Mason, J.H.	1936	Ibid , VII, 433
Martin, L.A.	1938	C.R.Soc.Biol.Paris, 127, 1059. (Vet.Bull., 9, 11.)
Mandia, J.W., and Bruner, D.W.	1951	J.Immunol, 66, 497.

MacLennan, A.P., and Rondle, C.J.M.	1957	Nature, 180, 1045.
McEwen, A.D.	1926	J.Comp. Path., 39, 253.
Meyer, K.F.	1915	J.Inf.Dis., 17, 458
Metaxa (quoted by Fried Berger and Frohner, 1910)	(1816	Cited by Stableforth and Galloway, 1959.
Miyasaik, S. and Hiraidi, I.	1957	Jap. J.Microbiol. 1, 37.
Mohan, R.N., and Bhadury, S.K.	1947	Ind.J.Vety.Sci. 17,247.
Mohan, K., and Hadish, S.M.	1966	Ceylon Vet. J., 14 , 52.
Mohteda, S.N.	1947	Ind.J.Vet.Sci., 17, 249.
Moussa, R.S. &	1959	J. Path. and Bact., 77, 341.
Mura, D.contini, A.	1951	Atti, Soc. ital.sci.vet., 5,280 (Vet Bull, 23, 349.)
Mukkur, T.K.S., and Nilakantan, P.R.	1968	J. Bact., 96; 2190
Naidu, P.M.N.	1937	Administrative Report of Mysore Civil Veterinary Department, (1937-38). Cited by Iyer and Ramchandran (1954).

Nangia, S.S., Baxi, K.K., Gularajani, T.S., and Seetharaman, C.	1966	Ind. Vet.J., 43,1279.
Nangyalai, M.A.	1969	M.Sc. thesis Magadh University.
Newsom, I.E., and Cross, F.	1933	Vet. Med., 28, 16.
Nikiphorova, N.M.	1953	Inst.Vet.Preparatov, 4, 338. (Vet. Bull., 26, 1134.)
Nikiphorova, N.M.	1958	Buff.Off.int. Epiz, 50, 176.
Nilakantan, P.R.e	1965	Ind.J. Vet.Sci., 35, 135.
Nitta, N.	1918	J.Comp.Path., 32, 122.
Omar, A.R., Phay, Cheah.Phee and Shanta, C.S.	1962	Brit.Vet.J., 118, 71.
Osler, A.G., Strauss, J.H., Mayer, M.M.	1952	Amer.J.Syph.Gonor and Ven.Dis., 36, 140. Cited by Bain (1961).
Otten, L. (Cited by Schutz, H.)	1939	Brit.J.Exp..Path,, 20, 235.
Pegreffi, G., Quesada, A., and Papandrea, E.	1951	Atti. Sci. ital, soci.Vet., 5, 280. (Vet. Bull., 23, 348.)
Perreau, P.	1960	Bull. Off. int. Epiz, 53, 116.
Perreau, P., Patit, J.P., and Thom M.	1964	Review Elov.Med.Vet., 17, 186. (Vet.Bull., 36, 863)

Piercy, S.E.	1951	British, Vet.J., 107, 65.
Pirosky, I.	1938a	C.R.Soci.Biol., 127, 98 (Vet.Bull., 8, 712.)
Pirosky, I.	1938b	Ibid, 127, 966. (Vet.Bull., 9, 168.)
Pirosky, I.	1938c	Ibid, 129, 346 (Vet.Bull., 9, 403.)
Priestely, F.W.	1936a	Brit.J.Exp.Path., 17, 374.
Priestely, F.W.	1936b	J.Comp.Path., & 49,340.
Rajagopalan, V.R.	1941 & 1942	Cited by Dhanda et al. (1956).
Rajagopalan, V.R.	1947	J.Path.Bact., 59, 37-50.
Rao, R.D.V., and Shammugham, S.	1964	Indian Vety.J., 41, 82.
Reichels, J., and Schneider, J.E.	1932	Amer.Vet.Med.Ass., 81,768.
Robertson, Muriel, and Felix, A.	1930	Brit.J.Exp.Path., 11,1&.
Roberts, R.S.	1931	J.Comp.Path., 44, 246.
Roberts, R.S.	1933	J.Comp.Path., 46, 56.
Roberts, R.S.	1947	Ibid., 57, 261.

Robinson, E.M.	1946	Emg. S. Afr., 21, 522. Cited by Stableforth and Galloway 1959).
Rotov, I.V.	1953	Veterinaria, Moscow, 32, 23. (Vet. Bull., 23, 448.)
Roux, E.	1888	Ann.de. Inst.Past, II, 49. Cited by Reichels and Schneider, (1932).
Sapre, S.N.	1945	Ind. J.Vet.Sci., 15, 151.
Schuitze, H.	1932	Brit.J.Exp.Path., 13, 285.
Scheuber, J.R.	1944	Onder Stepoort J.Vet.Sci.,19,17 Cited by Nilakantan (1965).
Sen Gupta,K.G.,and Verma, N.S.	1951	Ind.J.Vet.Sci., 21, 197.
Seetharaman, C., and Sinha, K.C.	1963	Vet. Biological product and their use, Indian Council of Argicultural Research,Animal Husbandry Series No. 2, New Delhi.
Shamatava, V.P.	1958	Veterinaria, Moscow, 35, 47. (Vet Bull., 29, 2299.)
Shirlaw, J.F.	1957	Brit.Vet.J., 71, 89.
Shrivastava, S.C.	1962	M.Sc. thesis Magadh University.
Singh, N.	1948	Ind.J.Vet.Sci., 18, 77.
Stableforth, A.W., and		

Stableforth, A.W., and Galloway, I.A.	1959	Infectious Diseases of Animals, Diseases due to Bacteria. Butterworths Scientific Publications, Vol. I & II, pp. 160 &413.
Sterne, M., Thorold, P., and Scheuber, J.R.	1951	J.Comp.Path., 61, 150.
Suchaci, I., Nedelcin, D., Ursache, R.	1956	Anu.Inst.Serurivacci, Pasteur, Bucuresti, 1, 211. (Vet. Bull., 27, 508.)
Thomas, J.	1968	Kajlan.Vet.Malaysia, Singapore, 1, 152. (Vet. Bull., 39, 4084.)
Tomic, L., Forsek, Z., and Romic, Z.	1960	Vet.Arhiv, 30, 49. (Vet. Bull., 30, 3525)
Thorold, P.W.	1953	Brit.Vet.J., 109, 302.
Tultsochinskaja-Tkalzchenko.	1934	Sovyet Vet., 10, 46. (Vet. Bull., 5, 472.)
Ubeev, A.D.	1958	Veterinariya Moscow, 35, No.11, pp 20.
VanSaceghem, R.	1938	Bull.Soc.Path.exot., 31, 472. (Vet.Bull., 9,11.)
Verma, N.S., Rau, K.G., and Votil, J.L.	1953	Indian Vet.J., 30, 33.
Viswanathan, G.R.	1934	Report of the Vet.Investigation Officer, Madras - 1935 Cited by Iyer and Ramchandran (1954).

Vittoz, R., Nguyem-Ngoc-Minoh.	1939	Rec.Med.Vet.exot., 12, 147.
Weberd, Bindrich.	1934	Z.Infektkor, Hanstiere, 46, 15. (Vet.Bull., 5, 115.)
Weinberg, M., and Mihailesco, M.	1929	Ann.Inst.Pasteur, 43, 1408. Cited by Stabeforth and Galloway,
Wells, E.A.	1962	Brit. Vet. J., 118, 336.
Westphal, O., Luderitz, C., and Bister, F.	1952	Z.Nature forsch, 7B, 148 Cited by Dhanda (1960).
Yusef, H. S.	1935	J.Path.Bact., 41, 203.

*****
***
*

Vittoz, R., Nguyem-Ngoc-Minch.	1939	Rec.Med.Vet.exot., 12, 147.
Weberd, Bindrich.	1934	Z.Infektkor, Hanstiere, 46, 15. (Vet.Bull., 5, 115.)
Weinberg, M., and Mihailesco, M.	1929	Ann.Inst.Pasteur, 43, 1408. Cited by Stabeforth and Galloway,
Wells, E.A.	1962	Brit. Vet. J., 118, 336.
Westphal, O., Luderitz, C., and Bister, F.	1952	Z.Nature forsch, 7B, 148 Cited by Dhanda (1960).
Yusef, H. S.	1935	J.Path.Bact., 41, 203.

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