Haemorrhagic Septicaemia

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HAEMORRHAGIC SEPTICAEMIA

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Front Cover Page Photo:

Terminally ill calf showing oedema, in recumbency

Back Cover Page Photo:

Healthy Buffaloes

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PREFACE

Haemorrhagic Septicaemia (HS) is an acute, fatal septicaemic disease primarily affecting cattle and buffaloes. The disease is caused by P. multocida belonging to serogroup B in Asian countries and India or E in Africa. The disease occurs as catastrophic epizootics in almost all parts of the world. Significant research achievements on various aspects of the disease have been made during the last 125 years not only in India but also in all parts of the world where the disease is prevalent. The Indian Veterinary Research Institute also has a long history of research on Haemorrhagic Septicaemia. In 1903, Dr Alfred Lingard a noted medical scientist and founder Director first produced anti-HS hyperimmune serum at Imperial Bacteriological laboratory (IBL), Mukteswar (now IVRI) and found it protective for susceptible animals against virulent challenge. Lieut.-Col. (then Capt.) J.D.E. Holmes, Bacteriologist at IBL, then improved the procedure for preparing the serum. During this period, about 3000 liters of immune serum was annually produced in Mukteswar for use in India.

This book on Haemorrhagic Septicaemia is a compilation of latest scientific information on every aspect of the disease. The book focuses on the epidemiology of the disease including its global occurrence & occurrence in India, economic losses, seasonal incidence & host susceptibility. The book also throws light on the Aetiology of Pasteurella multocida B: 2 (morphology, cultural and biochemical characteristics, host range, habitat and transmission channels, resistance, cellular components: Virulence factors, Antigens and Immunogens), symptoms in various animals, pathogenesis, diagnosis (provisional diagnosis, confirmatory diagnosis, laboratory procedures, differential diagnosis & choice of diagnostic test) alongwith treatment & prevention and control of the disease. Information about the history of vaccines & vaccination of HS, vaccines in current use, quality control of vaccines and the vaccination regime is also provided. The immune response to HS including naturally acquired immunity, humoral and cell mediated responses, immune response to vaccines & research methodologies for conventional and novel vaccines is discussed. The future perspectives of host-pathogen relationship and pathogenesis of P. Multocida, research on vaccine development & development of alternative and in vitro tests for potency evaluation has also been described in the book.

The defined goal of the book is to develop an information resource on the HS disease and is intended to supplement and complement the work of a large number of veterinary scientists, and research publications on various aspects of bacteriology, immunology, medicine and, information on therapy and vaccines that are currently available against the disease. The book is targeted to serve the veterinary students, practicing veterinarians, scientists, vaccinologists and policy makers to provide the scientific information regarding the various facets of the Haemorrhagic Septicaemia.

We hope that the book becomes a significant contribution to the existing knowledge base of Haemorrhagic Septicaemia disease and serve multiple roles, including that of a textbook, a review and a laboratory manual.

AUTHORS

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1. INTRODUCTION

Pasteurella multocida is a species of Gram negative bacteria belonging to genus *Pasteurella* which is a member of family *Pasteurellaceae.*

P. multocida is a heterogeneous species that includes strains of various serotypes with significant differences in biochemical composition of cell-wall structures which are accompanied by distinct variations in virulence, antigenic and serological, characteristics. Variations with respect to the virulence, pathogenicity for different animal species and outcome of infection are the most striking features of this heterogeneity.

The organism has been classified by various typing systems. Since specific serotypes of the organism can usually be correlated with host-species affected and virulence or disease (Table 1), serological typing has more utilitarian value than the other systems that are based on minor biochemical differences or DNA homology. Two serotyping systems are applied, which identify 5 serogroups (A, B, D, E, and F) and 16 serotypes (1 through 16) on the basis of differences in capsular and somatic antigens, respectively. Currently accepted practice encompasses both of these systems and *P. multocida* isolates are designated by stating the capsular group first, followed by the somatic antigen type (for example, A: 1; B: 2 etc).

The organism is a versatile pathogen. It has been recovered from over 40 species of domestic and wild animals and more than 100 species of birds. Under natural conditions, the organism causes a number of potentially fatal (*multocida*: fatal for many) and economically important clinical syndromes commonly known as Pasteurelloses. Various pasteurelloses include localized infections of various organs such as upper respiratory tract (Atrophic rhinitis and Snuffles), lungs (pneumonia), mammary gland and skin and, generalized fatal septic shock with multiple organ failure, such as Haemorrhagic septicaemia (HS) and Fowl cholera (FC).

Pasteurelloses can be grouped as primary and secondary for convenience. Bronchopneumonia in cattle (or Bovine Pneumonic Pasteurellosis or Bovine Enzootic Pneumonia or Shipping Fever complex or Bovine Respiratory Disease Complex, BRDC; Dabo *et al*., 2007), sheep, goats, pigs, and rabbits (Snuffles); wound infections in dogs, cats, and human; mastitis in cattle and sheep are the major examples of secondary Pasteurelloses. Secondary Pasteurelloses include diseases in which more than one serotypes of *P. multocida* (usually belonging to serogroups A) are incriminated as playing a role in association with other respiratory bacteria and viruses, and/or stress.

Fowl cholera (FC), Atrophic rhinitis (AR) and Haemorrhagic septicaemia (HS) on the other hand, are the examples of primary pasteurelloses in which only a specific serotype of *P. multocida* is associated with the disease. No other serotype of the species or any other bacterial or viral pathogen is involved with the morbid process and the outcome of infection.

Haemorrhagic septicaemia (HS) is an acutely fatal septic shock of buffaloes and cattle caused by specific serotype of *P. multocida*. Pigs are also affected naturally but the disease in pigs appears to be misdiagnosed and under-reported in India. HS occurs as catastrophic epizootics in many Asian and African countries resulting in high mortality and morbidity. Most Asian countries, including India, rank HS as the most important bacterial disease of cattle and buffaloes since the disease inflicts considerable economic losses to the farmers. Some authors are of the opinion that farmers "fear" this disease while they view FMD and other major infections in Asia merely as "nuisance".

HS and Haemorrhagic septicaemia (FC) are the only Pasteurelloses against which effective vaccines, though with some limitations, are available. While, HS still persists as one of the most economically important diseases in India even after regular annual vaccination programmes, the etiological agent of the condition- *P. multocida* remains a "mystery organism", even after about 140 years of its discovery.

Table: 1. Diseases caused by *Pasteurella multocida* serotypes in various animal species

Animal species	Serogroups/serotypes of *P. multocida*			
	B: 2 / E: 2	Serotypes of A	D	F
Buffalo	Non-commensal; H.S. (Fatal septic shock)	Normal Upper respiratory tract (URT) inhabitant;	Not reported	Not reported
Cattle	Non-commensal; H.S. (Fatal septic shock)	Normal URT inhabitant; Pneumonic infections; Bronchopneumonia in cattle: (Bovine pneumonic pasteurellosis or bovine enzootic pneumonia or shipping fever complex or bovine respiratory disease complex); mastitis	Not reported	Not reported
Sheep	Not Confirmed	Normal URT inhabitant Pneumonic infections; mastitis	Not reported	Not reported
Goat	Not Confirmed	Normal URT inhabitant (Pneumonic infections)	Not reported	Not reported
Pig	Non-commensal; H.S. (Fatal septic shock); Under-reported	Normal URT inhabitant Pneumonic infections; usually recovered from AR	Normal inhabitant of URT; Atrophic rhinitis (AR); co-infection with *Bordetella bronchiseptica* is an important factor; expresses an exotoxin with demonstrable osteoclastic activity	Not reported
Rabbit	Fatal septic shock after experimental infection; highly susceptible but no reports of natural B:2 infection	Normal URT inhabitant; Pneumonic infections- "Snuffles"	An AR-like syndrome; co-infection with *Bordetella bronchiseptica* may be a factor	Occasionally
Guinea pigs	Relatively resistant to a fairly large dose of experimental challenge	Respiratory infections	Not reported	Not reported
Dog	Not reported	Not reported	Not reported	Not reported
Cat	Not reported	Not reported	Not reported	Not reported
Horses	No recent reports	Not reported	Not reported	Not reported
Poultry; Duck	Resistant	Normal inhabitant; Fowl cholera (FC); (Fatal septic shock); A: 1, A: 3 and A: 4 commonly implicated serotypes	Occasionally associated with AR	Occasionally
Turkey	Not Reported	Fowl Cholera	-	Respiratory infections; FC
Mice	Septic shock-like condition after challenge infection; highly susceptible (50% lethal dose ~1-20 viable organisms), but natural infections not reported	Septic shock –like condition after challenge; highly susceptible	-	-

2. THE DISEASE

Haemorrhagic septicaemia (HS) is an acute, fatal septicaemic disease primarily affecting cattle and buffaloes. Pigs are occasionally attacked; feral ruminants and wild herbivores may develop the disease naturally. The disease is caused by *P. multocida* belonging to serogroup B (in Asian countries and India) or E (in Africa). With rare exceptions, no serotypes of *P. multocida* other than B:2 have been found associated with cases of classical bovine and bubaline HS in India. Other type B strains (B:3, B:4, B:11, B:4) have been associated with sporadic outbreaks of disease, but pathogenicity cannot always be demonstrated experimentally.

After subcutaneous inoculation of a virulent B:2 strain, the infected susceptible hosts exhibit all symptoms of classical HS and die within a short period of time indicating that B:2 is the only etiological agent of the disease. Classical HS, therefore, is a primary pasteurellosis. Nevertheless, it has also become clear over the years that many infections with pasteurellae do not produce classical HS in cattle and buffaloes (for example serotypes of A, D, and F; *P. haemolytica*), and, that many cases with typical symptoms and lesions of classical HS are not caused by *P. multocida*. **To clarify the confusion, the disease is, therefore, defined in terms of naturally affected animal species and the causative agent**. In tropical Asia and Africa, classical HS denotes an acute infection, mostly of cattle and buffaloes, with high mortality in clinical cases which is uniformly caused by types B:2 or E:2 *P. multocida*. So, HS is a particular kind of pasteurellosis, just as typhoid is a particular kind of Salmonellosis.

Pathologically, classical HS is an endotoxin (Lipopolysaccharide) induced fatal septic shock. The incubation period of naturally occurring and experimental disease appears to vary from 2 to 5 days. Disease generally progresses in 3 stages, the durations of which may vary and overlap each other. Initially, there is an increase in body temperature which is followed by respiratory distress, nasal discharge and salivation. The terminal phase is characterized by recumbency, septicaemia, and death. Mortality may be up to 100%. Cases may be classed as acute, subacute and chronic, or according to the localization of major lesions as cutaneous or oedematous, pectoral and intestinal. The characteristic signs of the disease include oedematous swelling of head and neck, and hemorrhagic lymph nodes. Post-mortem findings vary considerably according to the type of disease.

HS is vaccine preventable. In most countries where HS is endemic, annual prophylactic vaccination is done for controlling the disease. The currently used vaccines containing killed HS-causing *P. multocida* with an adjuvant are satisfactory and stretegic vaccination programmes have resulted in substantial reduction in losses. However, HS bacterins do not induce an across-serogroup protective response and have a very limited utility. Research to improve the cross-protective capabilities of the vaccines is required so that a single prepartion can be used as an umbrella for protection against both B:2 and A serotypes of the organism.

The OIE or World Organization for Animal Health originally classified haemorrhagic septicaemia as a list B notifiable disease. OIE-List B included diseases that were considered to be of socioeconomic and/or public health importance within the countries and which were also of significance to the international trade in animals and animal products. Later, a single list of notifiable terrestrial and aquatic animal diseases was established to replace former Lists A and B. Currently, (in 2020), OIE List of diseases, infections and infestations in-force, includes HS under the category of 'Cattle Diseases and Infections'.

KEY POINTS

1. Haemorrhagic septicaemia is an acutely fatal disease of cattle and buffaloes. Pigs are also affected.
2. No serotypes other than B:2 and E:2 cause classical HS.
3. Currently available vaccines against HS are satisfactory but not ideal. Research efforts are needed to improve the vaccines.
4. HS was previously included in List B diseases by OIE.

3. EPIDEMIOLOGY

3.1 Global occurrence

Hemorrhagic Septicaemia occurs as catastrophic epizootics in almost all parts of the world except in Oceania, Australia and Japan where it has never been reported. On the basis of distribution of the disease, nations of the Asian continent have been identified in three distinct categories (FAO-WHO-OIE, 1994).

In Category A nations, the disease is reported to be **endemic** and is of utmost economic importance. In addition to India, Bhutan, China, Indonesia, Malaysia, Mongolia, Myanmar, Philippines and Sri Lanka are included in this category. In the Category B nations, although sporadic outbreaks of the disease have been suspected but its presence has not been confirmed by isolation of agent and serotyping. Kuwait and Qatar have been included in this category. In Category C countries which are spread throughout the continent and include Singapore, Hong Kong and Israel, clinical reports of the disease in the past are on record, but presently, these countries are free of the disease. The disease has never been reported from Jordan (Table 2).

Table 2. Occurrence of Hemorrhagic Septicaemia in Asian countries

FAO-WHO-OIE Category	Occurrence	Countries
A	Endemic	Bhutan, China, India, Indonesia, Malaysia, Mongolia, Myanmar, Philippines and Sri Lanka
B	Sporadic outbreaks not confirmed by isolation and serotyping	Kuwait and Qatar
C	Probably existed, now free	Singapore, Hong Kong and Israel
	Never reported	Jordon

3.2 Occurrence in India

From 1936 to 1944, there was an average of 700 reported outbreaks of HS and 40000 deaths per year in India. During the 1950s, the average reported deaths per year ranged from 30000 to 60000. But in the 1960s and 1970s this figure dropped to approximately 4000 per year, presumably due to improved control measures (FAO 1991; De Alwis, 1999). Based on epidemiological data, Dutta *et al*., (1990) reported that during 1950 and 1990, 46-55% of all bovine deaths were due to HS. The authors also estimated that from 1974 to 1986, HS accounted for 6.3 deaths per year for every 100000 bovine populations. This amounted to 58.8% of the aggregate of bovine deaths due to the five epidemic diseases FMD, rinderpest, black quarter, anthrax and HS. Before 1939, the corresponding figure ranged from 13.4 to 20.9%. An increase in economic importance of HS, in relation to the other four epidemic diseases, was clearly established during the period. According to the findings of other epidemiological investigations conducted during various years, HS was one of the most reported diseases in the country during 1991 to 2010 and was found to be the cause of maximum number of deaths during that period. The disease was prevalent in almost all states of India (NADRES- the National Animal Diseases Referral Expert System). Previously, Dutta *et al*., (*loc cit*), had evaluated the overall state wise relative risk due HS and found it to be highest in Manipur (18.57%) and lowest in Dadra and Nagar Haveli (0.03%).

In some other location-specific investigations, similarly conducted by Saini *et al*., (1991), Jindal *et al*., (2002), Verma *et al*., (2004), Ahmad *et al*., (2005), Kumar *et al*., (2006), Singh *et al*., (2007), and Sharma *et al*., (2007), outbreaks of HS with high case fatality rate have been reported in different states of India. During 2007-2011, the average number of HS infected cases reported by the Department of Animal Husbandry and, Dairying Government of India, were 2416 in cattle and 825 in buffaloes. Average number of deaths in the respective species were 677 (CFR 28.0) and 347 (CFR 42.0); (Table 3). The highest cases of cattle infection were reported from the eastern region (43.71%), followed by western (27.06%), southern (22.42%) and northern (6.81%) regions. The

incidence pattern of HS in buffaloes was however different; it was highest in southern region (69.93%), followed by northern (20.97%), western (8.51%) and eastern (0.58%) regions. Recently, on the basis of about 82440 entries submitted to NADRES during the period 2016-17, Hemadri *et al*., (2017) reported that HS was one of the most prevalent diseases in the country occurring in about 20 states of India.

Table 3. Morbidity, mortality and Case Fatality Rates due to Haemorrhagic septicaemia during 2007-2011*.

Animal species	Population (in lakhs) (2007)	Average number of animals		Morbidity per million	Mortality per million	CFR (%)
		Infected	Died			
Cattle	1990.75	2416	677	12.14	3.40	28.0
Buffalo	1053.43	825	347	7.83	3.30	42.0

2* Basic Animal Husbandry Statistics, Govt. of India (various issues)

The incidences of morbidity and mortality during 2007-2011, as reported by the Government of India, appear to be comparatively lower than those estimated to occur during 1974-1986 by Dutta *et al*., (1990). The GOI projections for this period are most likely a significant underestimate of the disease incidence due to under-reporting and non-reporting of outbreaks (Singh *et al*., 2014).

3.3 Economic losses

HS is one of the most economically important diseases in South-East Asia. Estimation of actual economic losses due to HS is, however, a difficult task. Direct loss due to mortality is the first parameter that is taken into account for such estimations. But, depending on animal husbandry practices of locality and disease-reporting system of nation, many variables are involved and a wide discrepancy is bound to exist between the actual and reported deaths. Moreover, as losses are not merely due to the market value of the animal at the time of death, many other indirect economic parameters like the productive potential of the animal, its reproductive capacity and, in the case of draught power loss, the cost of alternate sources of draught power also need to be taken into account.

By using field survey data, Singh *et al*., (2014) developed a methodology for the evaluation of economic loss due to HS in India. The investigators included all the possible direct and indirect losses in cattle and buffaloes into account for computation of overall loss. The investigation revealed that the morbidity loss accounted for 23 per cent of the total losses incurred due to HS. Reduction in growth and, loss in milk production accounted for about half and one-fifth of loss respectively due to HS morbidity. Remaining (77%) losses were attributed entirely to mortality of the animals. The investigators estimated total economic loss per infected cattle as INR 6816 and as INR 10901 per buffalo.

All other available information on economic losses in South and Southeast Asian nations, in which HS is prevalent, indicates that the actual losses are considerably higher than the values computed in the respective studies.

3.4 Seasonal incidence

All tropical countries experience the greatest incidence of HS during rainy season, although sporadic cases may occur at any time during the year. Massive outbreaks of the disease have been reported in Sri Lanka (Perumalpillai and Thambiayah 1957; Dassanayake 1957), Sudan (Mustafa *et al*., 1978) and in Zambia (Francis *et al*., 1980) in rainy season. Seasonal incidences have also been well documented in India, with the majority of outbreaks occurring during the wet season (from July to September), with the peak in August.

Several factors have been associated with the seasonality of occurrence, but none of them explains the phenomenon satisfactorily. Physical stress of animals is considered one of those factors and increase in work performed by the draft animals in the rainy seasons is thought to be an

important cause. But, non-working and female animals are equally affected. Changes in nutrition, management and temperature with the changing season are thought to have some influence, but never been proved. Hot-humid climate is thought to promote the infection, but grazing cattle in the very dry regions of Equatorial Africa show naturally acquired immunity although clinical HS is unknown.

3.5 Host susceptibility and age

3.5.1 Cattle and buffaloes

In Southeast Asia, the major animal species susceptible to natural infection are cattle and buffaloes. It is generally accepted that buffaloes are more susceptible to *P.multocida* B:2 infection than cattle (De Alwis, 1999). Morbidity is higher in buffalo herds and mixed herds having high buffalo population. In India, Ramarao *et al.,* (1991) recorded a higher incidence of HS in districts where the buffalo population was higher. No striking differences have been observed within breeds of cattle and buffaloes in Asia.

There is ample evidence that in both species, young animals are more susceptible than older animals. The most susceptible age group in both species is between 6 months to 24 months. Singh *et al.,* (2014) have determined that about 75% of the total economic loss due to HS in India are attributed to young calves whereas; the contribution of adults is only about 25%.The animals of less than 6 months of age are generally immune due to presence of anti-*P.multocida* maternal antibodies transferred through colostrum, and incidences of HS deaths in adult animals are very low.

3.5.2. Pigs

Although the pig is not considered a conventional host, it displays susceptibility to HS-causing *P. multocida*. Incidences of septicaemic pasteurellosis due to type B infection in pigs have been reported in Malaysia and India (FAO 1959, 1979; Murthy and Kaushik 1965; Pillai *et al*., 1986). Gamage *et al*., (1995) reported an outbreak of acute septicaemic pasteurellosis in pigs in a farm in Sri Lanka, caused by serotype B:2. Verma and Sexena (1987) and Verma (1988) clearly established that septicaemic pasteurellosis in pigs in India was caused by serotype B:2. In 2009, type B:2 *P. multocida* was isolated form heart blood of pigs dying with symptoms and post-mortem lesions of septic shock (Rawat, unpublished data), and was named as strain “Soron”/ Subsequently, the strain was found to reproduce the condition in young piglets with classical symptoms, terminating in death after about 40 hours of experimental challenge. Typical post mortem lesions were observed and the organism could be re-isolated from heart blood as a pure culture (Verma, 2013).

3.5.3 Goat and Sheep

Although Pasteurellosis is an economically important syndrome of goat and sheep, the species do not appear to be affected by HS (B:2 infections) under natural or experimental conditions, as no outbreaks of B:2 associated HS like disease have been reported in these animal species in India and other Asian nations. The predominant organism that causes pasteurellosis in goats and sheep is *Pasteurella* (*Mannheimia*) *haemolytica*. Biotype A causes pneumonia in all ages of sheep, and septicaemia in young lambs. Biotype T, on the other hand, is associated with a distinct septicaemic syndrome in young adult sheep.

Goat do not appear to be particularly susceptible to either natural or experimental infection with Asian serotype B:2. Although no specific studies have been conducted in India, investigations carried out in Sri Lanka have provided conclusive evidence of the low susceptibility of goats to HS-causing *P. multocida*. No natural transmission occurred when goats were housed in close contact with clinically affected buffaloes and the animals did not even elicit an antibody response. Only 10% of the goats subjected to intranasal and subcutaneous inoculation with doses in the range as high as 10^{10} to 10^{12} colony forming units (CFU), died. Others showed merely a transient rise in temperature and swelling at the site of inoculation (Wijewardana *et al*., 1986b). Further, in an abattoir study, in 254 cases no carrier animals could be found (Wijewardana *et al*., 1986c). Thus,

there is no epidemiological or experimental evidence to incriminate the goat as a susceptible host or a reservoir of infection for the B:2.

Similarly, the Egyptian Rahmani breed of sheep was found resistant to infection with HS-causing *P. multocida*, as an isolate of the organism that consistently produced classical fatal HS in calves, failed to induce the condition in all experimental infections made by the subcutaneous or intranasal routes in sheep (Barakat *et al*., 1976).

3.5.4 Equines

Babes and, later, Lignieres (1887) described a bipolar bacillus, (named as *Bacillus equisepticus*) as the causal agent of equine influenza, and its association with that disease was frequently confirmed/ Subsequent investigations supported the view that cases of "pure haemorrhagic septicaemia" do occur in equines/ It was agreed that the disease runs a very acute course, illness lasting only for 1 or 2 days; the symptoms are fever, loss of appetite, restlessness or signs of colic, and occasionally subcutaneous oedema along the sternum and abdomen. Characteristic lesions are found on post mortem examination and the bipolar organism is present in large numbers in blood and body tissues. Subcutaneous inoculation of culture causes a hot, painful, oedematous swelling at the site and death within one to three days. HS has also been reported in horses and donkeys in India (Pavri and Apte, 1967) and Belorussia (Gevendze, 1987).

3.5.5. Other animal species

HS has also been reported in wild elephants in Sri Lanka (De Alwis and Thambithurai 1965; De Alwis 1982a; Wickremasuriya and Kendaragama 1982), in deer (personal unpublished data, Jones & Hussaini, 1982, Carrigan *et al*., 1991), bison (Chengappa and Carter, 1977); and antelope.

KEY POINTS

1. Haemorrhagic septicaemia is an economically important disease of global importance.
2. Major losses occur due to very high mortality among affected animals. The disease is the cause of highest cases of mortalities in cattle and buffaloes in India. In pigs, disease appears to be under reported.
   - Elephant, deer, bison and antelope are also affected by HS.
   - Goat and sheep are not affected by B:2 infections in natural conditions.
3. Young animals are more susceptible than older animals.
4. In India, highest incidence of HS occurs in rainy season. Sporadic cases may be found at any time during the year.

4. AETIOLOGY: *Pasteurella multocida* (B:2)

4.1 Morphology, cultural and biochemical characteristics

HS-causing B:2 strains of *P. multocida* are Gram negative, non-motile and non-spore forming coccobacilli to rod-shaped cells, measuring 0.3–1.0 µm in width and 0.6 – 2.0 µm in length (Fig. 1). Some organisms from freshly isolated cultures tend to stain dark at the poles and show bipolar staining. The bipolar characteristic is more conspicuous in blood films and tissue impression smears stained with Leishman or methylene blue stain. Repeated sub-cultures or cultures grown under un-favourable conditions tend to be pleomorphic and longer rods and filamentous forms appear. Strains may possess a demonstrable capsule. The capsule is usually seen only in freshly isolated cultures or in tissue or blood smears. It can be lost after repeated *in vitro* culture.

The organism is a facultative anaerobe. It has non-fastidious nutrient requirements and grows on most ordinary media like Nutrient agar. For routine primary isolation from clinical specimens, 5% sheep blood agar is the most convenient medium where good growth is obtained after incubation at 35-37^{0}C for 18-24 hours.

Fresh cultures of B:2 strains give typical fishy smell that can be identified by experienced workers. B:2 isolates produce small, colonies with bluish or yellowish-green tinge after overnight incubation (12-16 h) at 37^{0}C on blood agar (Fig. 2). Colonies may vary in size depending on the degree of capsulation. In broth media (Nutrient or BHI), floccular deposit with slight turbidity is produced after incubation. Special media such as dextrose-starch agar and casein-sucrose-yeast (CSY) medium support an abundant growth and are generally used for production of large quantities of antigenic biomass for vaccines.

Clinical Bacteriologists and Vaccinologists **do not utilize the Adansonian Concept of Taxonomists**, in which each characteristic is given equal weight. For simply confirming the identity of a pathogenic/vaccine strain of B:2 *P. multocida*, only 6 major characteristics (Table 4) are important. For detecting minor variations in field isolates, other biochemical tests can be conducted (Table 5). The variations are important only when they can be correlated with pathogenicity in animals.

As all other members of the *P. multocida*, B:2 strains are also oxidase and catalase positive**.** They have fermentative metabolism and produce acid in various carbohydrates. All strains produce Indole. Indole production is one of the most important and stable characteristic of *P. multocida.* Like all other serotypes, B.2 strains also fail to grow on McConkey's agar even after prolonged incubation for up to 5 days. This is an important characteristic that differentiates *P. multocida* strains from *Pasteurella* (*Mannheimia*) *haemolytica.* Based on observations with P_{52} and P_{25}, Dhanda and Sen (1972) reported that the strains of *P.multocida* show variability in their capacity to ferment Xylose. The authors associated this distinctive characteristic with virulence of the organism. The avirulent, non-HS causing P_{25} was not found to ferment Xylose whereas, the consistently virulent P_{52} readily utilized the sugar to produce acid. But it was later observed that there is a high degree of genomic similarity between P_{25} and Asian type B isolates (Townsend *et al.*, 1996). It can therefore be presumed that the capability to utilize Xylose does not bear any relationship with the virulence of the organism.

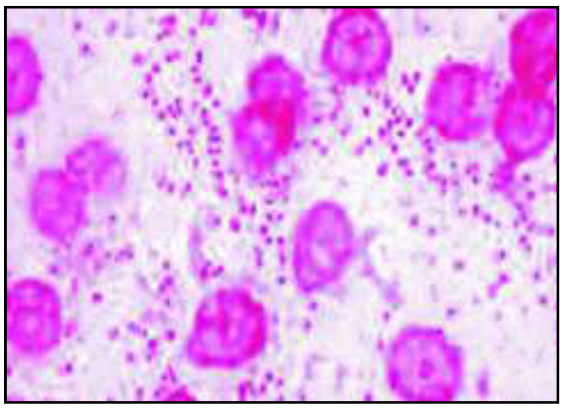

Fig. 1: Giemsa stained impression of spleen of mice challenged with *P. multocida*

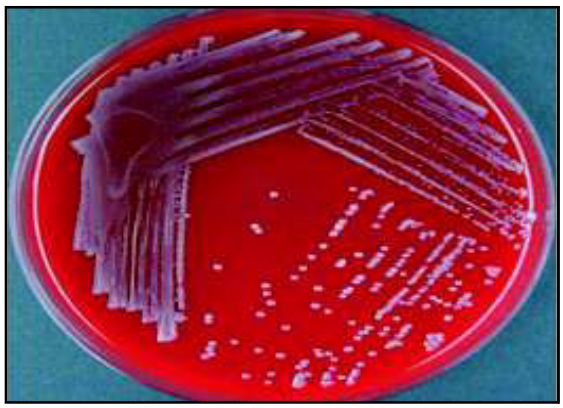

Fig. 2: Non-haemolytic colonies of *P. multocida* after 24h incubation at 37°C

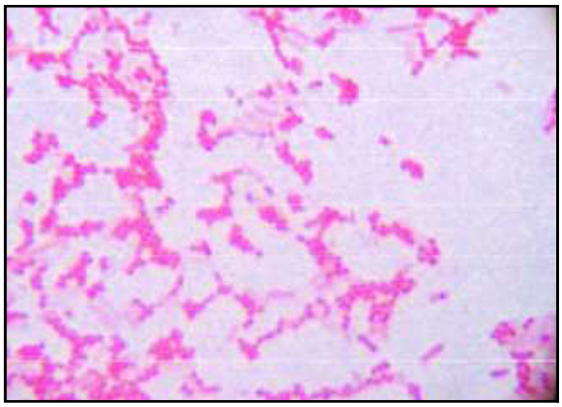

Fig. 3: Gram-stained preparation of *P. multocida*

Table: 4. Major tests for confirming the identity of the organism as *P. multocida*

Test	*P. multocida*
Haemolysis on 5% Sheep blood agar	Small non-haemolytic colonies
Gram's stain and morphology	Gram-negative; short coccobacillary rods
Motility	-
Oxidase (Disc)	+
Growth on MacConkey agar	No growth even after 5 days incubation
Indole	+

Table: 5. Sugar fermentation reactions of *P. multocida* $P_{52.}$

S. No	Test	*P. multocida*
1	Arabinose	+
2	Xylose	+
3	Adonitol	-
4	Rhamnose	-
5	Cellibiose	-
6	Nitrate reduction	-
7	H_2S production	-
8	Melibiose	-
9	Sucrose	+
10	Raffinose	-
11	Trehalose	-
12	Glucose	+
13	Lactose	+
14	MR	+

4.2 Host range

P. multocida B:2 is known to cause fatal infections in many animal species (*multocida*: fatal for many) but the host range of the organisms has not been investigated comprehensively using a number of strains. Although B:2 is not a host adapted pathogen, strains of the organism show a striking difference in their virulence against different animal species. The long-term epidemiological observations on natural incidences and experimental studies partly based on lethal doses (LD_{50}) have clearly established that the virulent strains of the serotype induce fatal shock in buffalo, cattle, pig, rabbit and mice. Sheep, goat, horse and guinea pig on the other hand, appear to be fairly resistant to both natural infections and experimental challenge. The virulence appears to be associated with absence of **Forssman antigen** (FA) in the tissues of an animal species. FA negative species are most susceptible to the infection (Table 6).

Moreover, as the natural incidence of the disease is nil in mice and very high in buffaloes, it is also very difficult to compare the relative susceptibility of mice (LD_{50}, 1-20 organisms; Rawat, 2018; Rawat and Tiwari, 2020) and buffaloes (10^4-$2x10^4$ organisms) by subcutaneous injection.

Table: 6. Relative virulence of HS-causing *P. multocida* for various animal species and occurrence of Forssman antigen in various tissues.

Animal species	Virulence	Forssman antigen (FA)
Rabbit	+++++	No
Mice	++++	Yes
Buffalo	++++	No
Cattle	+++	No
Pigeon	++	Yes
Pig	++	No
Horse	+	Yes
Goat	-	Yes
Sheep	-	Yes
Guinea-pig	-	Yes
Dog	-	Yes
Fowl	-	Yes (thymus and bursa of Fabricius
Duck	-	**Yes**

4.3 Habitat and transmission channels

The main sources of HS-causing *P.multocida* are blood and nasal secretions of terminally sick or dead animals. There are no permanent reservoirs of the organism outside host, but infected pasture, carcass and infected bedding may be the sources of infection, particularly in hot and humid climate. Though, *P. multocida* type B:2 is not considered to be a part of normal microflora of the upper respiratory tract of domestic animals, carrier status has been described by some workers. The organism is supposed to be transmitted by direct contact with infected animals and fomites.

4.4 Resistance

Although the organism is susceptible to mild heat (55°C) and most hospital disinfectants, it is commonly believed that it can survive for hours and probably days in humid soil and water.

4.5 Cellular components: Virulence factors, Antigens and Immunogens

Although the virulence factors of HS-causing *P. multocida* have been poorly elucidated (*refer* Chapter 11) it is known that for successful **invasion, survival, colonization,** and **spread in host animal**, B:2 strains express certain virulence factors that help in evading and modulating the actions of host immune system (Graydon *et al*., 1993).

Capsules, that basically have an antiphagocytic function; adhesins (type 4 pili and outer membrane proteins), that contribute towards colonization; hyaluronidase, that promotes

spreading; and iron-regulated and iron-acquisition proteins that are required for growth and survivability in the host environment have been identified as factors involved in processes essential to pathogenic lifestyle of the organism. In pathogenesis of HS, the specific or combined roles of these factors are however, limited only to the survival and multiplication of organism in hosts (Chung *et al.,* 2001; Harper *et al.,* 2006). The principal factor that is responsible for the septic shock and death of host is lipopolysaccharide (LPS).

4.5.1 Capsule

The capsule (or outer layer) of the cell is believed to be responsible for the serogroup specificity. The biochemical composition and size of capsule differ between serogroups, serotypes and strains of *P. multocida* (Rosner *et al.,,* 1992; De Angelis *et al.,* 2002).

B:2 strains produce small quantities of high molecular weight acidic polysaccharide capsule (De Angelis *et al.,,* 2002). Type A strains, on the contrary, produce capsule composed of hyaluronic acid (Penn and Nagy, 1976). According to the available information, polysaccharide capsule of B:2 is a surface antigen containing mannose, galactose and arabinose, which behaves as a hapten when injected in most of the animals. Capsular polysaccharides have been extracted and purified by various methodologies (Mukkur, 1971; Penn and Nagy, 1976; Giridhar *et al*., 1990), but stimulation of antibodies to highly purified capsular material was found difficult, possibly due to presence of large amounts of non-immunogenic proteins (Rimler and Rhoades, 1989). Purified polysaccharides being T-cell independent antigens induce only IgM type response without IgG class switch over. Therefore, protective role of purified capsule of HS-causing *P. multocida* remains doubtful even when significant antibody titers are detected against these polysaccharides.

4.5.2 Neuraminidase and hyaluronidase

Neuraminidase is produced by strains of all serogroups of *P. multocida*- A, B, D and E (Rimler and Rhoades, 1989). The enzyme is known to remove N-actylneuraminic acid (NANA) from a number of substrates of the host tissues and induce different biological effects that are believed to help in invasive processes of the organism during initial stages pathogenesis of HS**.** The substrates on which neuraminidase act, include mucin, fibrinogen, erythrocytes, heart cells and IgG. The enzyme is not considered to be the sole cause of disease or fatality.

The production of hyaluronidase and chondroitinase by HS-causing B:2 serotypes is also well documented (Carter and Chengappa, 1980; Rimler, 1993; Rimler and Rhoades, 1994). Hyaluronidases are enzymes that are normally associated with invasive mechanisms in bacteria. Whilst it may be concluded that hyaluronidase production is a characteristic exclusively restricted to serotype B:2 strains that cause HS**.** De Alwis (1996) described a type B:2 mutant that was of low virulence to mice and rabbits and avirulent to cattle and buffaloes but produced hyaluronidase. Therefore, no clear relationship has been established between ability to produce hyaluronidase or any other enzyme, and virulence.

4.5.3 Proteins

Outer membrane (OM) helps the bacteria to survive in the host environment by allowing selective transport of material through it. Approximately 50% of dry mass of outer membrane Gram-negative bacteria (GNB) consists of proteins, and more than 20 immunochemically distinct proteins have been identified (Henderson *et al.,* 1996). Hatfaludi *et al.,* (2010) have categorized OMPs of *P. multocida* on the basis of their functional characteristics as structural proteins, transport proteins adhesins, protein assembly systems, and membrane associated enzymes. These proteins not only have several important physiological and nutritional functions that help in multiplication and growth of HS-causing *P. multocida* within host tissues but, they also interact phenotypically with the host immune system during pathogenesis and immune response. Therefore, OMPs, especially those, that are surface exposed, may play a role in pathogenesis, and may be useful immunogens to be included as vaccine candidates (*refer* Chapter 11).

4.5.4 Endotoxin and lipopolysaccharide (LPS)

Endotoxins are invariably associated with Gram-negative bacteria as constituents of the outer membrane of the cell wall. Although the term endotoxin is occasionally used to refer to any "cell-bound" bacterial toxin, it is more commonly applied to describe the lipopolysaccharide complex (LPS) integrated with outer envelope of Gram-negative bacteria because the endotoxic activity of these bacteria is expressed when free, monomeric LPS is released from their OM either naturally, or through action of antibiotics (Shenep *et al.,* 1988). Structurally, all Gram-negative LPSs are composed of three covalently linked parts**:** Lipid A---Core polysaccharide--O polysaccharide (Figure 9). The toxicity of LPS is associated with Lipid A, and the immunogenicity is associated with O polysaccharide components.

Under normal physiological conditions, infecting live, intact Gram-negative bacteria continuously shed small amounts of OM in the hosts although the quantity of released LPS remains insufficient to induce any patho-physiological effect. But when LPS is released abruptly in sufficient quantities, it manifests its toxicity by inducing production of pro-inflammatory cytokines (TNFα, IL-1 and IL-6) which, depending on their quantity, initiate a series of progressive pathological changes resulting in fatal septic shock (Figure 4; *refer* section 6, Pathogenesis; Henderson *et al.,* 1996). Thus, LPS (endotoxins) significantly contribute to the symptoms of Gram-negative septicaemia and therefore represent the most important virulence factors in Gram-negative infections (Raetz and Whitfield, 2002). HS, caused by serotype B:2 of *P. multocida* can be considered as the classical model of LPS mediated fatal septic shock.

The LPS are complex molecules with molecular weights over 10,000 that vary widely in chemical composition both within and between Gram-negative groups. The basic architecture of LPS of different *P. multocida* types is similar to that of other Gram-negative organisms (Figure 9) but recent studies have elucidated that each of the 16 Heddleston serotypes expresses structurally distinct LPS molecules/ Each of Heddleston's serotype is capable of inducing protective immunity against the homologous serotype but cross-protection is rare. Some variations in chemical composition of O-polysaccharide units and KDO of *P. multocida* strain Pm 70 have been identified (St. Michael *et al.,* 2005) but no correlation either with toxicity or immunogenicity has been reported.

LPS appears to be the major antigen involved in serotype classification of *P. multocida*, but its role as an immunogen remains doubtful. Mice, cattle, and rabbits have not been readily protected against *P. multocida* challenge following immunization with B: 2 LPS. When purified LPS were used to actively immunize mice prior to challenge, results obtained by several investigators (Rimler *et al.,* 1984; Lugtenberg *et al.,* 1986; Ramdani and Adler, 1991; Lu *et al.,* 1991b; Adler *et al.,* 1996) were similar. Though the antibody titers to purified LPS were comparable to those of mice immunized with whole bacteria, LPS was found non-protective. Immunization with whole bacteria was always found to confer 100% protection. One interesting observation of Pati (1994) and Samanta (2001) is that the Westphal purified LPS of the Indian HS strain (B: 2; P_{52}) did not confer protection against homologous challenge, but on the contrary, increased the susceptibility of immunized animals as the mean death time of immunized mice groups was significantly less than that of un-immunized controls.

Whilst the presence of other virulence factors of B: 2 cannot be discounted, there is sufficient evidence to implicate LPS as a factor predominantly responsible for HS. The evidence supports its role in inhibiting phagocytosis, thereby enabling rapid multiplication, and further reinforces the role of the capsule in the virulence mechanism (resistance to phagocytosis is greater in encapsulated strains). It also accounts for the acute phase response (APR; Figure 9) and the typical lesions observed in HS.

5. SYMPTOMS

5.1 Buffalo and Cattle

On exposure to natural or experimental infection, clinical signs usually develop after a brief incubation period. Under natural conditions, the period of incubation in buffalo and cattle is probably from one to two days. Under experimental conditions, however, the initial symptoms marked by rise in temperature and development of a local swelling at the site of injection are generally observed in from six to eighteen hours after the inoculation of virulent culture through subcutaneous route. The progression of the clinical syndrome may broadly be divided into 3 phases.

The first phase is marked by the onset of the earliest symptom *i.e.* a rapid rise in body temperature to 41° or 42°C. Fever lasts throughout the course of the disease but drops to subnormal levels during the terminal phase, a few hours before death. Fever is usually accompanied by cessation of feeding and rumination and general depression.

The second phase is dominated by a respiratory syndrome. The pulse and respiration become accelerated, mucous membranes appear hyperemic and saliva flows from the mouth. At first there is constipation, but is soon followed by diarrhea, with signs of colic and straining; later the fluid faeces may contain flakes of mucus or blood. Submandibular oedema may also begin to show during this phase.

The subsequent course of disease which is characterized by recumbency (Fig. 4) varies in different breeds of animals, outbreaks and locations, and several forms have been observed and described. The respiratory distress becomes more acute, the animal lies down, terminal septicaemia sets in and death follows: case fatality is nearly 100%. According to duration of progress, cases may be classed as peracute, acute, subacute and chronic; or according to the localization of major lesions as cutaneous or edematous, pectoral and intestinal. In many instances, there are varying degrees of overlap.

Figure 4 Terminally ill calf showing oedema, in recumbency
(About 48 hours after challenge with 50 million mouse lethal dose of P_{52}).

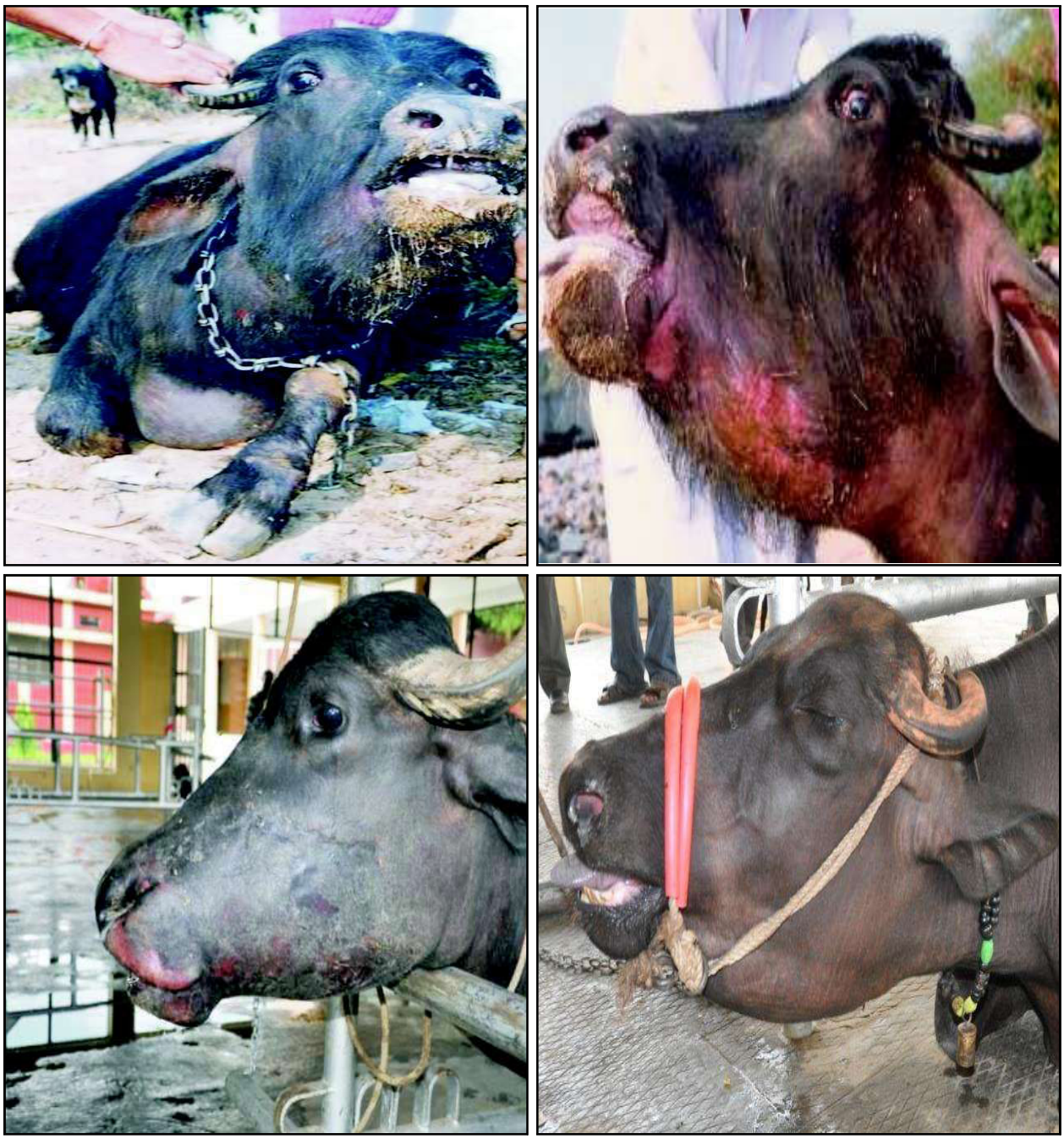

Fig. 5 (A,B.C,D): Buffalo calves affected with HS showing submandibular oedema and respiratory distress

5.2 Pig

Three well defined pasteurelloses *viz.* Atrophic Rhinitis (AR), pneumonic and septicaemic pasteurellosis are known to occur in pigs. AR is a primary pasteurellosis caused particularly by capsular serotypes D and is associated with Pasteurella multocida toxin (PMT). It is a localized condition of nasal turbinate bones. Pneumonic pasteurellosis of swine is usually caused by type A and D (White *et al.,* 1993). Septicaemic pasteurellosis of pigs is caused by B:2. The clinical manifestations of the B:2 infections in pigs are similar to those commonly found with classical bovine and bubaline HS. Both in naturally occurring cases (Radostis *et al.,* 1994) and in experimentally infected animals (Verma, 2013; Figures 5, 6, and 7), fever, dyspnoea, lameness, oedema of throat and lower jaw, subcutaneous hemorrhages, recumbency and death are observed. In young piglets, per-acute deaths without any clinical symptoms may occur within a period of 12 hours.

Fig. 6: Pig; 24 h after challenge

Fig. 7: 48 h after challenge

Fig. 8~60 h after challenge

5.3 Mithun

Outbreak of HS has also been reported in Mithun.

A **B**

Fig. 9: Terminally ill Mithun (A) showing extensive oedema (B) recumbency and epistaxis.

KEY POINTS

1. In naturally occurring cases caused by B:2 strains, terminal septicaemia is the main characteristic feature in all forms of the disease.
   - In peracute cases, sudden death without clinical signs may be observed.
   - Acute disease generally progresses in 3 phases with symptoms of fever, respiratory distress with nasal discharge, and frothing from the mouth, leading eventually to recumbency and death.
   - Severe subcutaneous oedema of the mandible, neck and brisket is a distinctive feature of the disease.
   - Case fatality may reach 100 percent. Mortality is largely confined to older calves and young adults.
2. Water buffaloes show more severe forms of disease than observed in cattle. Pigs also show classical signs of HS after experimental infection.
3. Serotypes A infections predominantly show clinical signs of pneumonia and death.

6. PATHOGENESIS

For convenience, the pathological sequence of events which may be correlated with the 3 phases of progression of clinical symptoms (*refer* section 5), can also be divided in 3 phases.

In natural cases of HS, during the initial phase, the organisms possibly enter the host through the tonsillar region where they invade tonsils via macrophages and multiply rapidly in peritonsilar soft tissue. Like all *P. multocida*, HS-causing strains are also capable of rapid multiplication once established in soft tissue. The infected animal mounts an inflammatory response to the local infection which is characterized by onset of fever and associated symptoms.

If the phagocytic system of animal fails to the arrest the overwhelming local multiplication of bacteria, macrophages carry viable organisms to local lymph nodes where they multiply and spread to surrounding tissues and, invade circulation and other body organs. The bacteraemic phase of pathogenesis is marked by skin (submandibular oedema) and respiratory symptoms.

The terminal phase, which is marked by recumbency and death of animal, is the result of a complex series of pathobiological effects known as septic or endotoxaemic shock. The most important event which triggers septic shock in susceptible animal species (cattle, buffalo, rabbit and mice) but not in resistant species (Sheep, goat, guinea pig, dog, cat) is large-scale intravascular lysis of *P. multocida* with abrupt release of huge quantities of free LPS and endotoxin. Free LPS then manifests its toxicity by inducing release of pro-inflammatory cytokines (TNFα, IL-1 and IL-6; Henderson *et al*., 1996) which, depending on their quantity, induce a cascade of progressive morbid changes resulting in fatal septic shock (Figure 10).

Septicaemia is essentially a terminal event and pasteurellae can only be isolated from blood during the terminal phase of the disease. At the time of death, around 10^5-10^6 CFU/ml of organisms appear in the blood. The observation clearly indicates that release of free endotoxin is an intravascular event. While clinical signs of shock may be replicated in cattle and pigs with endotoxin alone (Rebers *et al.,* 1967; Rhoades *et al.,* 1967), they are manifested in the terminal stages of the disease. So it is unlikely that endotoxin contributes significantly to the initial pathogenesis. Some investigators are however, of the opinion that the entire clinical syndrome is due to the effects of endotoxin alone (Horadagoda *et al.,* 2001).

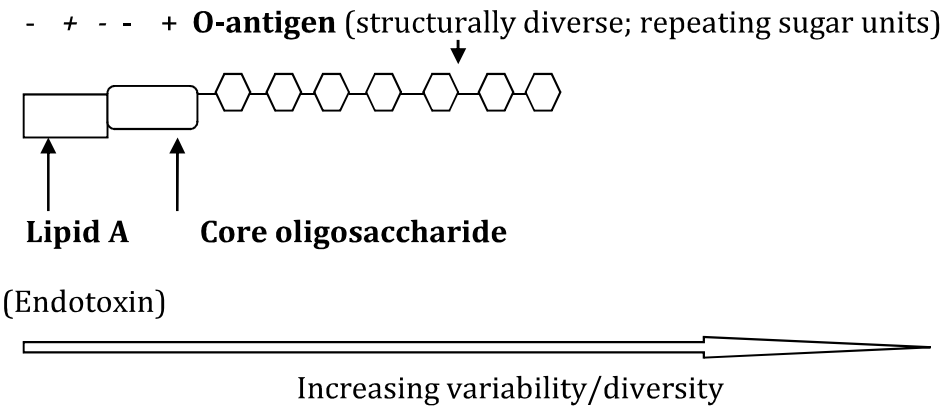

Fig. 10: Schematic illustration of the basic architecture of lipopolysaccharide (LPS) from Gram-negative bacteria

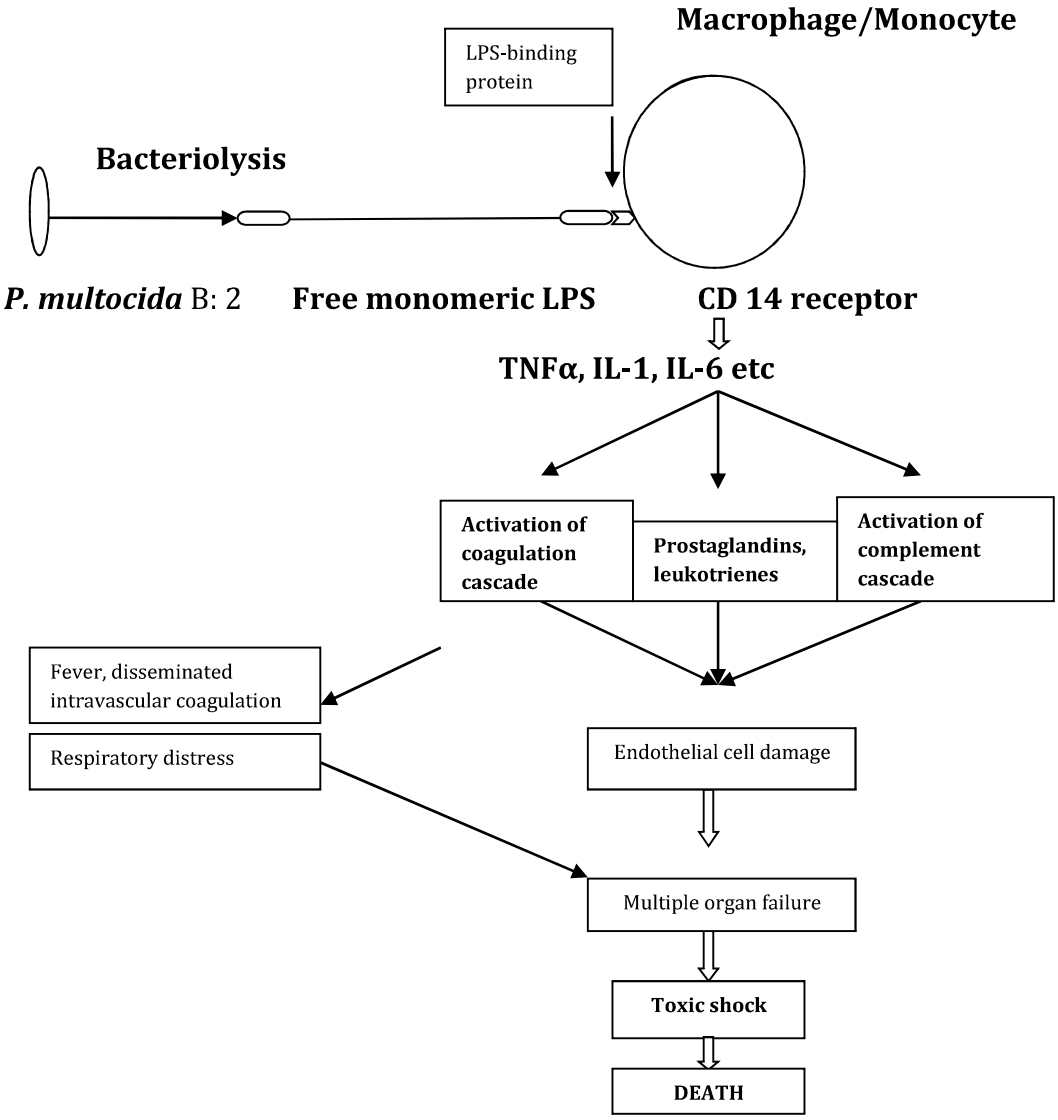

Fig. 11: Pathophysiology of lipopolysaccharide (LPS; endotoxin)-mediated septic shock (HS)

KEY POINTS

1. Capsule, hyaluronidase, neuraminidase, and outer membrane proteins promote initial growth, multiplication and haematogenous spread of organism within hosts.
2. Fatal endotoxaemic shock results from over-expression of cytokine-mediated host response to free LPS liberated during the bacteraemic phase.
3. Factors involved in intravascular bacteriolysis of HS-causing *P.multocida* in susceptible hosts still remain unknown. The organism remains a pathological mystery.

7. DIAGNOSIS

7.1 Provisional diagnosis

Since immediate preventive measures are required to control the spread of HS outbreaks, a quick presumptive diagnosis without waiting for laboratory confirmation is always desirable. This can be readily done on the basis of clinical signs, post-mortem lesions and history of occurrence of the disease.

Where oedema of the throat is present (Fig. 4, 5), the disease can be diagnosed on this general clinical ground only. But, as outlined in section 5, most cattle and buffalo cases are found with a variety of clinical signs, none of which, when considered individually, is specific for HS. Infectious diseases, such as Shipping fever, Anthrax, Black Quarter and Pneumonic pasteurellosis caused by other serotypes are often confused with cases of HS. The most important post-mortem lesions suggestive of HS are subcutaneous oedema (Fig. 12) and petechial haemorrhages, particularly on the base of the ventricle. **Lung lesions are less dominant in cases of HS**; if present, congestion, gradual consolidation and thickening of the interlobular septa giving rise to lobulation, may be found. For making a provisional diagnosis, the entire clinical picture should be taken into consideration and should be interpreted along with the pathological findings and available epidemiological data.

7.2 Confirmatory diagnosis

After making provisional diagnosis, the disease can be confirmed in laboratories on the basis of isolation and identification of the causative organism. Isolation and identification of the causative agent from the terminally affected/ dead animals is the basis of diagnosis of HS, and **wherever possible, cultures should always be made so that the causative strain may be serotyped**.

7.2.1. Preferred clinical specimens:

Heart blood or visceral organs (spleen, liver) of recently died animals yield *P. multocida* readily in pure cultures on blood agar. If facilities are available at the location of the carcase, a post-mortem examination should be carried out. The gross pathological lesions should be observed at this time and, blood should be aseptically drawn directly from the heart, immediately. Alternatively, blood may be obtained by puncture of the jugular vein. In an outbreak, it is always desirable to collect material from more than one animal.

Bacteriological examination of material collected from clinically affected/suspected HS cases before death may not give consistent results. In some instances however, ante-mortem testing of nasal secretions may yield virulent pasteurellae upon culture. Animals almost always die after establishment of bacteraemic phase. Since, bacteraemia is established during the terminal stages of disease shortly before death (*refer* section 6; Pathogenesis), and much of the bacterial multiplication takes place in the carcase after death, blood will give positive cultures only in the terminal stage immediately before death. Care must be taken to avoid collecting material from animals treated with antibiotics.

For isolation from decomposed carcasses, bone marrow or brain samples are preferred. The bone marrow is usually the last tissue to become invaded by post-mortem contaminants and is the best source material in an animal dead for more than a few hours.

7.2.2. Dispatch of diagnostic specimens

Blood and/or tissue specimens should be packed on ice or placed in a suitable transport medium (Annexure-2). Recovery of pasteurellae from field specimens is improved by use of a transport medium. If this is not possible, the samples should be dispatched at room temperature to the laboratory as quickly as possible. Long bones should be cleaned, and sent to the laboratory with minimum delay.

7.3. Laboratory procedures

P. multocida is a biohazard Risk Group 2 organism (OIE, 2008). For routine diagnostic work which has been described below, clinical specimens and suspected or confirmed isolates may be handled in a Biosafety Level 2 laboratory with modest facilities.

7.3.1. Diagnosis based on culture and identification

7.3.1.1. Microscopic examination

Although not very useful due to presence of contaminating organisms, a microscopic examination on clinical specimens should always be conducted (Annexure-2). *P. multocida* can usually be found in smears from the blood or pathological exudates, such as oedema fluid, pericardial effusion, and in spleen impression smears. Microscopic examination of Wright's or Giemsa stained preparations from dead animals may reveal capsulated rods with typical bipolar staining (Fig. 1).

7.3.1.2. Bacteriological media and isolation

If the clinical material is uncontaminated, colonies can be isolated on a direct culture plate within 24 hours. Primary isolation can be routinely done on 5% sheep blood agar. However, any other suitable medium that is able to support growth of pasteurellae, especially when only a few are present, may also be used. For example, Tryptose-tryptone agar containing 0.1% sucrose and 0.1-0.3% yeast extract (Annexure-2) may be used because the medium permits colonies to develop from very small inocula. Examine for colony and growth characteristics after incubation.

If the clinical material is contaminated, isolation of *P. multocida* becomes very difficult because the contaminants overgrow the organism. In such cases, inoculation of material in mouse with subsequent isolation from heart blood of dead mouse is most satisfactory (Annexure-3; A.1)

7.3.1.3. Examination of colony characteristics

On media containing 5% sheep blood, non-haemaolytic colonies are produced after 24 h of incubation at 37˚C (Fig. 2). The colonies may show different size, consistency and colour depending on the serotype. Select representative colonies to make Gram-stained smears and, examine under microscope (1000x) to detect presence of organisms morphologically indistinguishable from *P. multocida* (Fig. 3; Annexure-2).

Conduct an oxidase test (Annexure-2) on representative colonies. A positive oxidase reaction differentiates the organism from most of other Gram-negative organisms belonging to various genera. Further, selected colonies may be subjected to a rapid slide agglutination test (Fig. 13; Annexure-2) using anti-*P.multocida* (B:2) hyperimmune serum for making a rapid diagnosis. The serum may be raised in-house by the laboratory in rabbits.

On the basis of colony characteristics and direct microscopy of culture, positive oxidase and positive slide agglutination tests, a positive report may be issued by the laboratory within 24 hours.

7.3.1.4. Conventional biochemical tests for identification

Although shared by all *P.multocida* in general, some reactions are almost always constant among HS-causing strains. Possession of **oxidase** and **catalase**, formation of **indole** and fermentation of **glucose, fructose, mannose, galactose and sorbitol** (a great majority of strains ferment xylose and sucrose) along with **absence of motility** and **inability to grow on McConkey's** agar confirm the identity of an isolate as *P. multocida* beyond doubt. Therefore, a Gram-negative, coccobacillary, non-motile organism that produces oxidase, catalase and indole; grows readily on sheep blood agar and produces small non-haemolytic colonies, but fail to grow on McConkey's agar even after prolonged incubation can be identified as *P. multocida***.** Additionally**,** young cultures on a solid medium give a typical fishy smell that can be easily identified by experienced workers.

7.3.2. Serotyping

From an epidemiological standpoint and for the purpose of initiating control measure in an outbreak, it is important to know the serotype. For veterinary bacteriologists, the serological classification systems appear to be more useful than the other systems that are based on minor biochemical differences or DNA homology, because they usually correlate specific serotypes or strains with host-specificity, virulence, or disease (Table 1). Therefore, a *P.multocida* isolate needs to be further characterized for its serotype.

Several serological classification systems for *P.multocida* have been developed previously. In the currently accepted system, serotypes of are identified on the basis of differences in capsular and somatic antigens. Two serological methods are used to type isolates**.**

7.3.2.1. Indirect Haemagglutination Assay (IHA) for capsular typing

The first method, developed by Carter (1955) is based on passive or indirect hemagglutination test that utilizes erythrocytes sensitized with capsular antigens. It recognizes five serogroups (A, B, D, E, and F). The sixth group (C) shown in Table 1, is not a valid serogroup since strains belonging to this group are non-encapsulated. In the indirect haemagglutination (IHA) assays, the surface antigen of the organism is liberated by mild heat treatment and the supernatant, which contains the antigen, is used to coat erythrocytes. Agglutination of the sensitized erythrocytes against serial 2-fold dilutions of hyperimmune rabbit antiserum is considered positive. A positive control may be set up using a known reference culture to prepare antigen and coat the cells. An uncoated erythrocyte suspension is used as the negative control. The serum used is the same as for the rapid slide agglutination test.

The original tests described by Carter (1955) used human 'O' erythrocytes obtained as outdated cells from blood banks as well as fresh cells obtained from a donor. Subsequently, a modified version of the test using formalinized human 'O' RBCs was described by Carter and Rappay (1962) and Carter's group recommended the use of formalinized RBCs, as those could be kept in cold storage for 12 months. Later, Sawada *et al*., (1982) used glutaraldehyde-fixed sheep erythrocytes (sRBCs) for IHA. The IHA test using heat-extracted capsular antigen is suitable only for serotyping and is not used for assessment of immune response of animals.

7.3.2.2. Agar Gel Precipitation (AGPT) test for somatic typing

The second method, developed by Heddleston and co-workers in 1972, is based on agar gel precipitation test (AGPT) that utilizes heat-stable somatic antigens (i.e. expressed lipopolysaccharides) obtained by heating at 100°C. Their anti-*P.multocida* sera were prepared in chickens and, finally 16 types were identified. Therefore, the typing system recognizes 16 serotypes that are designated by numbers 1 through 16.

The currently accepted serotyping system for *P.multocida* encompasses both of these serological tests, and designates the capsule type stated first, followed by the LPS type. (e.g., a designation of A:1;3 is capsular type A, Heddleston LPS types 1, and 3).

7.3.2.3. Non-serological tests for typing

Although the Carter and Heddleston scheme is the standard serotyping system, the methods are time consuming and require availability of specific typing antisera. This makes the system un-practicable for routine diagnostic purposes. Several non-serological tests have therefore been developed for routine determination of specific types of new *P. multocida* isolates. Group D strains of *P. multocida* can be identified by Acriflavine test. Type A strains produce hyaluronic acid capsule. Thus, hyaluronidase-producing *Staphylococcus aureus* strain can be used to identify mucoid type A strains by hyaluronidase decapsulation test. Similarly, a hyaluronidase production test can be used for identification of type B strains that cause HS (Annexure-3).

7.3.3. Enzyme-linked immunosorbent assays for diagnosis

Dawkins and associates developed an ELISA for rapid identification of HS causing *P.multocida* in Australia in 1990. In the assay, rabbit anti *P. multocida* immunoglobulin (Ig) fractions

were used for coating, and different dilutions of 'boiled antigens' of 124 strains, consisting of 58 reference strains and 66 field isolates from collections of various laboratories were tested (*see* Annexure-3 for procedure). The assay showed specificity of 99% and sensitivity 86%. However, due to low sensitivity and development of polymerase chain reaction (PCR) based molecular diagnostic tests, ELISA-based tests are not presently used for diagnosis of disease and also not recommended by OIE (Kharab, 2015). The technique may be more useful for screening a large number of cultures from a collection, rather than occasional diagnosis of HS from specimens.

7.3.4. Genetic typing methods:

Considering the difficulties associated with the serological typing methods, a number of genetic typing methods have been developed. Polymerase Chain Reaction-based assays have been designed to distinguish *P. multocida* from other bacterial species (PM-specific PCR; Townsend *et al.,* 1998 and 2000), to differentiate strains from each of the *P. multocida* capsular serotypes, and to detect HS-causing serotype B:2.

The PM-specific PCRs are based on primers specific for the *kmt1,* and 23S rRNA genes. Both PCR assays amplify a DNA product from all *P. multocida* strains, including strains from each of the *P. multocida* subsp. *multocida*, *gallicida*, and *septica.* The primer pair KMT1SP6-5'-GCTGTAAACGAACTCGCCAC-3' and KMT1T7-5'-ATCCGCTATTTACCCAGTGG-3'specifically amplifies a product of approximately 460 base pairs (bp) in all subspecies of *P. multocida* and in *Pasteurella canis* biovar 2.

The Capsular multiplex PCR assay uses five primer pairs (Table: 7), each specific for sequences within the capsule biosynthetic locus of the different capsular serotypes. These assays allow discrimination of all the capsular types (A, B, D, E, and F) but are not phenotypic tests. They may give a positive result for strains that do not express a capsular polysaccharide as a result of mutation.

Table 7. Primers for multiplex PCR for *P. multocida* capsular types

S. No	Capsular type	Primer name	Sequence of primer	Product size (bp)
1	**A**	CAPA-F	5'–TGCCAAAATCGCAGTCAG – 3'	1044
2		CAPA-R	5'–TTGCCATCATTGTCAGTG – 3'	
3	**B**	CAPB-F	5'-CATTTATCCAAGCTCCACC-3'	760
4		CAPB-R	5'–GCCCGAGAGTTTCAATCC–3'	
5	**D**	CAPD-F	5'-TTACAAAAGAAAGACTAGGAGCCC-3'	657
6		CAPD-R	5'-CATCTACCCACTCAACCATATCAG-3'	
7	**E**	CAPE-F	5'-TCCGCAGAAAATTATTGACTC-3'	511
8		CAPE-R	5'-GCTTGCTTGATTTTGTC-3'	
9	**F**	CAPF-F	5'-AATCGGAGAACGCAGAAATCA-3'	851
10		CAPF-R	5'-TTCCGCCGTCAATTACTCTG-3'	

7.3.4.1. PCR assays for B: 2-specific identification of *P.multocida*

P. multocida serotype B:2-specific primers HS-B2-F and HS-B2-R (Table 11) designed by ICAR-Indian Veterinary Research Institute, Izatnagar (HS-PCR; AINP on HS, 2016), can be used for specific detection of the HS-causing serotype. The expected amplicon size for B:2-specific primers is ~320 bp. In addition, serogroup B cultures with any combination of somatic antigens of *P.multocida* can be identified by the amplification of a 560 bp fragment with KTSP 61 and KTT 72 (Table 11). Type B PCR is 100% specific for HS-causing B serotypes (Townsend *et al.,* 1998 and 2001).

For conducting PCR assays, bacterial DNA, bacterial colony or field samples such as nasal swabs, morbid materials like heart blood, spleen or bone marrow can be used as template. The procedural details of these PCR assays have been given in Annexure-3.

In addition to the PCR-based tests, 2 other methods developed for analyzing DNA of HS causing *P. multocida* strains include Ribotyping and field alternation gel electrophoresis (Adamson *et al*., 1993; Townsend *et al*., 1997b), and Restriction endonuclease analysis (Wilson *et al*., 1992; Rimler (1997). Analysis of the DNA restriction patterns using these methods has proved useful in differentiating bacterial strains that have been denoted as identical by all previous typing methods. But, such finer confirmations are not usually needed for routine diagnostic purposes, and the applicability of these tests as diagnostic tools remains limited.

7.4. Differential diagnosis

The HS can be confused with many diseases including shipping fever, anthrax, black quarter and even with Classical swine fever (CSF). The **Shipping fever** (Bovine Respiratory Disease Complex) is often confused with HS. But the disease has multiple etiologies, often involving *Mannheimia haemolytica*. *P. multocida* serotypes A and Respiratory Syncitial Virus (RSV). Additionally, shipping fever complex **is not a septicaemic disease**; multiple petechial haemorrhages and multiple organ failure are not usually found in clinical cases of the disease. **Pneumonic pasteurellosis** should also be considered for differential diagnosis. Isolation and identification of serotype involved help in differential diagnosis.

Anthrax and Blackleg: The per-acute nature of the disease and the extensive oedema and haemorrhages make it difficult to differentiate HS clinically from blackleg and anthrax. However, anthrax can be diagnosed quickly by microscopic examination of wet blood films, and blackleg can be differentiated on the basis of presence of muscle lesions having crepitating sound and foul smell.

Classical swine fever (CSF): Most of the symptoms of CSF in pigs resemble with the symptoms endotoxaemic shock. However, Differential Leukocyte Count (leucopoenia) can help in initial diagnosis of CSF. Isolation and identification of *P. multocida* from dead animals confirms septic pasteurellosis.

7.5. Choice of diagnostic test

A wide range of laboratory tests for biochemical, serotype and genotypic identification of *P. multocida* strains have been developed over the years. Although, conventional biochemical system in combination with serotyping serve the purpose of identification of HS-causing *P. multocida* quickly and satisfactorily, Polymerase chain reaction (PCR)-based assays designed to detect serotype B:2 *P. multocida* can also be used. However, such finer confirmations are not usually needed for routine diagnostic purposes, and the applicability of these tests as diagnostic tools remains limited (Table: 6). PCR-based and other genetic assays therefore remain an epidemiological and research tools for reference laboratories.

Table: 8. Choice of diagnostic tests and procedures for HS

S. No	Test/Procedure	Relevance as		Comments
		Diagnostic tool for HS	Epidemiological and research tool	
1	History of occurrence	++++	++++	For implementing immediate preventive measures, a **quick presumptive diagnosis** without waiting for laboratory confirmation is an absolute necessity during an outbreak; This can be readily done on the basis of these observations
2	Clinical signs	+++	-	
3	Post-mortem lesions	+++	-	
4	Microscopic examination on clinical specimens	++	-	
5	Mouse inoculation of contaminated specimens	++++	-	Mice screen contaminating organisms and *P. multocida* is isolated in pure cultures from heart blood of dead mice.
6	Culture and identification by conventional biochemical tests	++++	-	On the basis of colony characteristics, direct microscopy of culture and a positive oxidase and rapid slide agglutination test, a positive test report may be issued by the laboratory within 24 hours.
7	Serotyping on isolates by serological methods	++	++++	Time consuming; require availability of specific typing antisera
8	Serotyping on isolates by non-serological methods	+++	-	Help in rapid determination of serogroup. For making final diagnosis, history, clinical signs etc can be taken into consideration.
9	ELISA-based tests for identification	-	++	More useful for screening a large number of cultures from a collection, rather than occasional diagnosis of HS from specimens. With development of PCR based tests ELISA-based tests are not presently used for diagnosis of HS.
10	PM-PCR for detection	-	++	HS serotype-specific PCRs preclude the need to conduct PM-PCRs on clinical specimens for diagnostic purpose.
11	Multiplex PCR for detection of serogroups	+	+++	HS serotype-specific PCRs preclude the need to conduct capsular PCR on clinical specimens for diagnosis.
12	Serotype specific HS-PCR	+	+++	Detection of presence B: 2 in nasal specimens may prove helpful in identification of infected animals in early pyrexial phase, so that their treatment may be started.
13	Other molecular tests	±	+++	Useful in differentiating *P. multocida* strains.

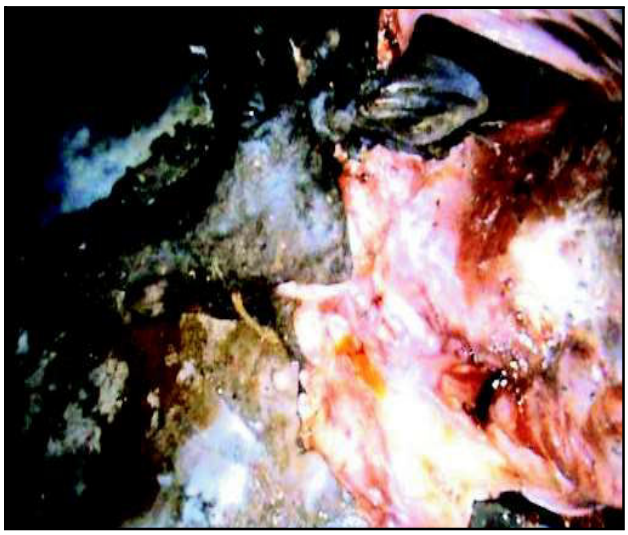

Fig. 12 Gelatinous oedema of neck

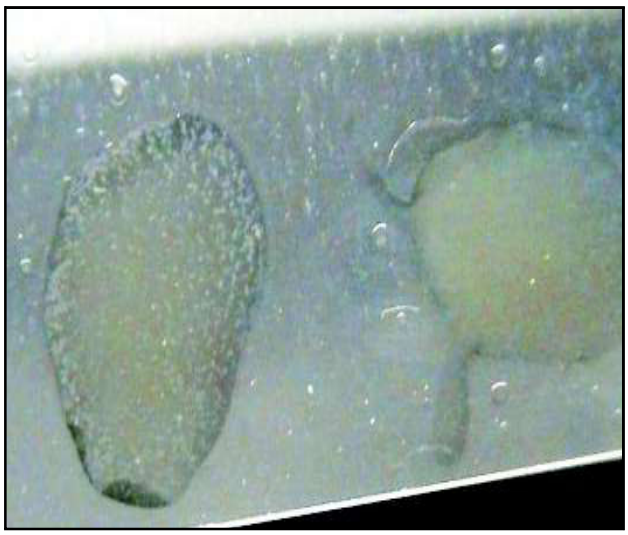

Fig. 13 Rapid slide agglutination test

KEY POINTS

1. At field level, an early tentative diagnosis based on history of occurrence, clinical signs and symptoms, and post-mortem lesions can be done by veterinary clinicians.
2. For confirmatory diagnosis which involves isolation, identification and serotyping, clinical samples should be sent to the nearest laboratories.
3. A wide range of laboratory tests for biochemical, serotype and genotypic identification of *P.multocida* strains have been developed over the years.
4. Differential diagnosis with Shipping Fever and other pneumonic pasteurelloses, Anthrax, Black Quarter, and classical Swine Fever is important.

8 TREATMENT, PREVENTION AND CONTROL

8.1. Antibiotic therapy

According to most of the earlier and recently published investigations conducted in various regions of India (Bandopadhyay *et al.,* 1991; Abeynayake *et al.,* 1993; Arora & Sharma 2015 and 2016; Roy 2015; Sharma 2015; Bhandari & Roy 2017) and the other parts of the world, strains of *P.multocida* appear to be susceptible to all commonly used antibiotics (penicillin, ampicillin, streptomycin, tetracycline, chloramphenicol, erythromycin, neomycin, sulfadiazine and sulfonamide-trimethoprim combination), and resistance to antibiotics is not a problem of any major concern till now. Additionally, many antibiotics, which include penicillin, amoxicillin (or ampicillin), cephalothin, ceftiofur, cefquinome, streptomycin, gentamicin, spectinomycin, florfenicol, tetracycline, sulfonamides, trimethoprim-sulfamethoxazole combination, erythromycin, tilmicosin, norfloxacin, enrofloxacin (and other fluoroquinolones) and amikacin have been reported to have some clinical efficacy against HS.

But, the major problem with the treatment of HS with antibiotic is that good success rate is seen only when the treatment is given at a very early stage of the disease. HS treatment is not successful when given in advanced cases. Investigators of all nations where HS is prevalent agree that only an early treatment may be successful, and nothing is of much avail in later stages of disease. Additionally, although not particularly documented for HS in cattle and buffaloes, there is considerable information from reports of field outbreaks indicating that antibiotic **treatment in the terminal stages accelerates death**. This is a phenomenon very similar to the commonly reported complication of antibiotic therapy of humans suffering from Gram-negative sepsis. This happens because administrations of antibiotics to human or animal host with septicaemia precipitates an endotoxin shock due to rapid release of free LPS and endotoxins from the lysed bacteria (Shenep *et al.,* 1988). Thus, detection of cases in early stage of HS appears to be the key of success of any antibiotic therapy.

In practice, however, as the disease occurs mainly in situations with primitive husbandry practices, most field cases escape detection in the early stages. This usually renders treatment ineffective. In organised farms, on the other hand, a practical method of achieving early detection and successfully treating animals is to check the rectal temperatures of all in-contact animals regularly once an outbreak has been detected. Any animals showing an increased temperature can be separated and treated with a course of an appropriate antibiotic. Specific detection of B:2 serotype in nasal specimens may prove helpful, but due to acuteness of the disease, practicability of using the HS-specific PCR test in field or farm conditions remains to be explored.

An antibiotic sensitivity test on clinical isolates of *P. multocida* is desirable. But due to acuteness of the disease, it becomes impracticable. Therefore, prior information on usual patterns of antibiotic sensitivity in the area/farm is of immense help in selection of antibiotic. In general, of the antibacterial compounds that are recommended for use in cattle and buffaloes, and that can be conveniently and economically administered, penicillin, ampicillin and oxytetracycline appear to be the most useful.

8.2. Serum therapy

Lignieres and Spitz were first to show the prophylactic and therapeutic efficacy of polyvalent immune serum against HS in 1902. They prepared the polyvalent serum in horses by injection of strains of organisms from six species of animals which was used both as a prophylactic and for the treatment of the disease. In 1903, Alfred Lingard hyper immunized cattle and buffaloes at Imperial Bacteriological Institute, Mukteshwar, and found their serum protective for susceptible animals in doses of 5 to 10 c.c (ml) against an injection of virulent culture. Later, Holmes of the same Institute, improved the procedure for preparing the serum. During this period, about 3000 liters of immune serum was annually produced in Mukteshwar for use in India.

A brief description of the entire method of serum production adopted at Mukteshwar is: Cattle and buffaloes were first injected subcutaneously with a protective dose of immune serum.

Four hours later, the animals received 0.1 c. c. (ml) of forty eight-hours grown broth culture of virulent HS bacilli. An injection of a dead vaccine, followed in 10 days by 0.1 c.c. virulent culture was also used for initial protection of animals in place of immune serum. The second injection of the virulent culture was given with an increased dose of 1 ml after 8 to 10 days when the temperature reaction and local oedema following the first inoculation subsided. Further injections with gradually increasing doses of culture from 5 c. c. to 500 c.c. (ml) were given at intervals of 10 days. Finally the animals were bled for serum. The injections and bleedings were repeated, and the dose of culture was gradually increased up to 1000 c.c.

An injection of the antiserum conferred immediate immunity that lasted for a period of 3 to 4 weeks. The serum was found very effective when used during HS outbreaks; the mortality being at once arrested. During the same time various workers employed serum simultaneous method of immunization in which cattle and buffaloes were immunized with injection of immune serum along with virulent culture. Holmes (1907) also used this method but owing to the risk involved, the method was not employed in India. Dhanda *et al*., (1959) subsequently produced more useful protective serum by injecting cattle and buffaloes with several doses of oil adjuvant vaccine followed by virulent bacteria.

Later, production of hyperimmune haemorrhagic septicaemia serum in India and South East Asia declined greatly. Currently, serum therapy is only of theoretical interest. Kheng and Phay (1963) used 60-100 ml of hyperimmune serum for experimental administration to two-year-old buffaloes at varying periods from six hours before to 18 hours after infection without obtaining any significant therapeutic effect.

8.3. Prevention and control

Vaccination is the most important, if not the only, measure that has been found effective in controlling the disease in countries where it is endemic. Details of vaccines in use and vaccination procedures are given in chapter 9. Preventive measures recommended by Mosier (1993) and De Alwis, 1999) have been described here.

8.3.1. In endemic areas/countries

1. Vaccination should be routinely practiced in endemic areas / countries just before the beginning of rainy season.
2. An effective reporting system should be established; in most endemic countries, including India, HS is a notifiable disease.
3. Over-crowding and mixing of animals from endemic and non-endemic areas should be prevented, especially during hot humid climate and rainy season.
4. Additionally, farmers should be trained to recognize signs of disease, and to dispose carcasses of dead animals properly.

8.3.2. During outbreaks

1. In situation of an outbreak, vaccination programme should be continued. The formalin inactivated bacterins with alum adjuvant provide protection for ~6 months. The formalin inactivated bacterins in oil adjuvant are thought to provide protection for 12 months. As an early protective response against infection is desired, during outbreaks, broth bacterins or the alum precipitated (or aluminium hydroxide gel) vaccines are preferred. However, plain broth or alum adjuvanted bacterin, and the oil adjuvant vaccine may be administered at different sites simultaneously.
2. Field level veterinary clinicians should carryout post-mortem examinations wherever possible, collect and dispatch clinical specimens to nearest diagnostic laboratory immediately, and should make a tentative diagnosis based on previous history, clinical symptoms and post-mortem examinations.

3. Veterinarians should also make efforts to isolate suspected animals in very early stage of the disease, and treat them with a broad spectrum antibiotic. This is very difficult for free-range animals but may be done relatively easily in organized farms, where the rectal temperature of all in-contact animals can be taken.
4. Farmers should be trained to recognize early signs of disease, to dispose carcasses of dead animals properly and to dispose unconsumed fodder and other materials from infected premises.
5. Deep burial or incineration is recommended for carcasses. Fodder etc should be disposed within the premises by deep burial, drying and burning. Effluents from sheds should be prevented from being washed away from the infected premises. Drains carrying such material should be led into a deep protected pit within the premises or subjected to disinfectant treatment.
6. Over-crowding should be avoided especially during hot humid climate and monsoon season.
7. Additionally, movement of herds in and out of a diseased premises or villages should be restricted as much as possible.

8.3.3. Prevention of spread across borders

1. Across territory transportation and mixing of animals from endemic to non-endemic area should be restricted. Only the animals from a region where no outbreaks have occurred for a minimum period of one year should be allowed to mix.
2. Animals from a farm or herd of origin and / or other in-contact animals should be randomly blood tested to detect presence of antibody titers by indirect haemagglutination test. Presence of high IHA titers is an indication of recent exposure to disease and therefore the presence of HS-causing organism in the locality.
3. Before transport, animals should be kept under observation for 2 to 3 weeks. Such animals should be tested repeatedly for detecting presence of *P. multocida* in nasal samples and for IHA antibodies in serum.
4. After transport to the new location, animals should be quarantined for 2 to 3 weeks. During this period bacteriological examination for detecting presence of *P. multocida* in nasal samples, and IHA to detect serum antibodies should be done repeatedly.

KEY POINTS

1. Antibiotic treatment of HS is successful only when given at a very early stage of disease. Treatment given in later stages may accelerate death.
   - In conditions of primitive husbandry practices, it is very difficult to detect field cases in the early stages.
   - In organised farms, early detection of suspected animals for treatment may be done by checking the rectal temperatures of all in-contact animals regularly, during an outbreak.
2. Antibiotic sensitivity test on isolates is desirable but, penicillin, ampicillin and oxytetracycline appear to be the most useful and can be used routinely.
3. Serum therapy, once used as an effective method for control of HS outbreaks, is not practised these days.
4. Vaccination is the most effective control measure in all countries where HS is endemic.
5. For prevention, several measures are adopted according to the situations existing in a particular country or area.

9. VACCINES AND VACCINATION

9.1. History and development of various types of vaccines

A wide variety of vaccines have been developed and tried in past and new improved vaccines are in process of development with the objective to provide a long-lasting protective response against the disease.

The first vaccine against HS was probably developed as early as in 1887 by Oreste and Armanni, who attenuated *Bacillus bubalisepticus* by passage through pigeons. Oreste and Morcone (in 1889) then used cultures of the attenuated organism for immunization of buffaloes and sheep after growing the organism at a temperature of 30 to 32°C. Many investigators then tried avirulent and attenuated living vaccines but none of those vaccines became established in practice because of difficulties in preparation and distribution.

Later, studies on vaccines containing dead organisms gained momentum and Holms (1910) tested the immunity conferred by dead vaccines prepared in various ways from cultures of the organism. Broth culture of a virulent strain of organism heated to 65°C for 30 minutes and then Carbolized was found to protect young cattle and buffaloes when injected subcutaneously with a dose of 5 to 10 c.c., for a period of six weeks against challenge with virulent culture. The vaccine produced a protective response on 4th day of inoculation. In India, about 150,000 doses of this vaccine were produced and employed annually as a prophylactic measure in enzootic areas during 1920s.

Later, a broth vaccine containing Alum precipitated inactivated *P. multocida* cells, probably developed by an American Commercial Manufacturer prior to 1950, was used in Philippines. The vaccine was shown to produce immunity against challenge for up to 6 months. The efficacy of the vaccine was found to be associated with the dry mass of the bacteria (2.0 mg /dose) and concentration of alum adjuvant (1%). The Alum precipitated vaccine was also employed extensively in southern India (Iyer *et al*., 1955), Central Africa and USSR. Since the broth based alum precipitated vaccine was not found free from shock reactions, Aluminium hydroxide gel based vaccine was developed and Thailand began using the vaccine from 1979. Though the duration of immunity imparted by gel vaccine is similar to that of the alum precipitated, the incidences of shock reaction were greatly reduced with gel vaccine.

The concept of using liquid paraffin and lanolin emulsion as an adjuvant (lipovaccines) was tested as early as in 1916, in France. A small amount of experimental vaccine against HS was made in Burma in 1952, using formalin inactivated *P. multocida* recovered from an ox and emulsified in liquid paraffin and lanolin. Since the results of this vaccine in experimental animals were excellent, further tests were conducted using cultured *P. multocida* organisms. Depending on the results of the preliminary trials conducted in Thailand, the dose decided was not less than 2.0 mg dry mass of phase-I *P. multocida* in a volume of 3 ml oil emulsion. In India, Dhanda *et al*., (1956) produced oil adjuvant vaccine (OAV) and demonstrated promise of immunizing cattle and buffaloes for about a year. But OAV was unpopular among the field workers because of its high viscosity and poor syrigeability (De Alwis, 1992). Thus a multiple emulsion vaccine having low viscosity was developed by various investigators in India (Mittal *et al*., 1979; Gupta *et al*., 1979; Yadav and Ahooja, 1983; and Uppal, 1983). The formulation was reported to confer a protective humoral immunity up to one year in calves (Verma and Jaiswal, 1996).

A live vaccine using a serologically related strain of serotype B: 3,4 from fallow deer was used to vaccinate cattle and was found to confer protection for one year, comparable to the duration of immunity (DOI) by OAV (Myint *et al*., 2005). However, this vaccine was not found suitable for primary vaccination of young buffaloes. Some attenuated vaccines of streptomycin-dependent mutants (Chengappa and Carter 1979; Dwivedi and Sodhi, 1992); temperature-sensitive (ts) mutants (Schimmel, 1993) and aroA mutants (Hodgson *et al*., 2005) have also been tried but danger of reverting back to pathogenic form always exists with these mutants.

9.2 HS vaccines in current use

Currently available Haemorrhagic septicaemia vaccines are preparations of an HS-causing virulent strain of *P. multocida* serotype (B:2). Pure, whole culture of the vaccine strain is inactivated by formaldehyde and a suitable adjuvant is added. The vaccines are used for the active protection of cattle and buffaloes against HS. In India, 3 types of HS vaccines are produced. Alum precipitated vaccine containing ~2 mg dry mass of bacteria with 1 percent alum/dose is thought to provide active immunity for ~ 5 months against challenge. HS Alum gel adjuvant preparations containing ~2.5 mg of killed antigenic bacteria/dose provide protection for 6 months. HS oil adjuvant preparations containing ~2.5 mg/dose of killed antigenic bacteria are known to provide protection for one year.

During 2018-19, approximately 75 million doses of HS vaccines were produced in India by State Biological Production Units and Private Manufacturers; out of which ~54 percent of doses were produced by State Biological Production Units (Tiwari *et al*., 2020). Bain *et al*., (1982) have determined that up to 5 million doses a year of oil adjuvant vaccine can be made by small-scale **cottage industry** methods using vortex tanks. For large quantities a **factory scale** approach is needed.

9.3 Vaccine manufacture

9.3.1. Vaccine strains

Most countries use their local isolates for production of HS vaccines. Bain (1979b) compared the protective efficacies of *P. multocida* cultures grown *in vivo* as well as *in vitro*, after propagation in bovine cells and laboratory subcultures on media. Active protection tests in mice using bacteria grown *in vivo* gave cross-protection between Asian strains (B: 2), African strains (E: 2) and the Australian strain 989 (B: 3,4), whereas laboratory subcultures gave only homologous protection. It is generally agreed therefore, that vaccines produced from fresh field isolates are more efficacious than those produced from seed cultures propagated *in vitro* in laboratories over long periods.

In India, a highly virulent *P. multocida* strain P_{52} (B: 2) of IVRI is used for production. The strain may be obtained from ICAR-Indian Veterinary Research Institute, Izatnagar.

9.3.1.1. Choice and selection of a vaccine strain:

A local/wild virulent strain of HS-causing *P. multocida* may be selected and used for vaccine production by the manufacturers if the strain is shown to be satisfactory with respect to safety and immunogenicity for each of the animal species for which the vaccine is intended. The selection however, must be based up on some desirable characteristics of the new strain.

There has been some speculation as to whether strains exist in nature that have special immunogenic properties. Arawwawela *et al*., (1981) and De Alwis (1984) conducted studies to evaluate comparative immunizing properties of *P. multocida* strains from different countries. The investigators produced suspension of each strain after growing under the same conditions. To examine cross-protective capability of the strain, mice groups were immunised with each suspension standardised on a dry weight basis. The results showed that although no consistent difference in protection was demonstrable when the mice were challenged with a local isolate, the density of growth, and therefore the dry weight yield of whole bacteria, varied between strains. It was concluded that strains that are well capsulated and express complete range of cell wall antigens give higher dry matter yields, and may therefore be better immunogenic strains for vaccine production. While selecting a new vaccine strain the above information is worth considering and, it is best to select an isolate of confirmed serotype identity, which has been tested and proven to be immunogenic in target animal species and which gives high dry weight yields.

9.3.1.2. Characteristics of strain P_{52}

P_{52} belongs to serotype B:2 and possesses all growth and metabolic characters of *P. multocida* (described in section 4.1). The strain grows readily on 5% sheep blood agar at 37 °C to produce small, non-haemolytic colonies with slightly bluish tinge within 24 h (Fig. 2). Young cultures of the organism emit a fishy smell which can be easily recognized by experienced workers. Morphologically, P_{52} is a small, Gram-negative, non-motile rod (Fig. 1). The organism is oxidase and indole positive, but it fails to grow on McConkey's agar even after prolonged incubation. It produces acid without gas in glucose, fructose, mannose, galactose, sucrose and xylose (Table 4).

P_{52} is highly virulent for rabbits, mice, cattle, and buffalo at very low doses and causes rapidly fatal infections in those species when inoculated through appropriate route. Guinea pig is resistant to a fairly large dose of the organism including P_{52} strain. Sheep, goat, dog, fowl, and duck have not found to be affected with fatal B:2 infections in field conditions, and the organism is **not known to cause disease of any significance in humans.**

9.3.1.3. Storage and maintenance of stock cultures

During storage, either in stock culture medium or in freeze-dried form, dissociation of P_{52} (and other B:2 field isolates) may take place giving rise to phenotypically and genotypically altered forms which are neither virulent nor immunogenic (Rawat, unpublished data). Such cultures are not suitable for production and potency testing of vaccine batches. Preservation and maintenance of seed stocks by the manufacturing units and testing laboratories are therefore of utmost importance. Thus, the selected strain should be passaged, preferably twice, in buffalo calf or male cow calf (target animal species, which is inexpensive). After the second passage, the strain should be re-isolated on Blood agar and freeze-dried (Annexure-3). It is very important to keep the production and challenge stock cultures in Phase I; i.e., the cultures should be kept within 1 or 2 transfers on blood agar from infected blood before lyophilization.

Being consistently virulent for cattle, buffalo and experimental animal species (mice and rabbit), P_{52} is also employed for challenge of animals during potency testing of HS vaccines in the India. Determination of mouse minimum lethal dose (MLD) of any strain used for challenge is important because when the potency tests are conducted on cattle and buffalo, the recommended challenge dose for these animals is equivalent to 50 million mice MLD**.** Though, it may be advantageous to have minimum lethal dose (MLD) values of the challenge strain for the animal species on which challenge test is to be performed, it is determined for mice due to economic and practical considerations.

MLD of P_{52} for mice has been determined several times in the Division of Standardization, IVRI (Rawat and Tiwari, 2019) and has been accepted as 0.2 ml S/C injection of 10^{-8} dilution of a young (12-16 h) growth of P_{52} obtained on BHI or Nutrient broth at 37°C. A local HS-causing strain of *P. multocida* shown to be consistently virulent for cattle and buffalo may also be used for potency testing, if it has been properly characterized in terms of its mouse minimum lethal dose (mouse MLD; Annexure-3).

9.3.2. Production process

A flow chart of the entire production process for HS vaccines is given in Fig. 14.

9.3.2.1. Dense cultures

Production of HS vaccine requires a dense culture of the organisms. For that, depending on scale of production, any one of the two culture methods may be employed. A suspension of any desired density can be made from washed off bacterial growth obtained on solid media in Roux flasks. But, for large-scale production, seeding solid media and harvesting growth from it becomes messy and uneconomical. The second method using liquid media in Fermenters is more practical and economical for the large scale production. Rani *et al.*, (2006) have investigated effects different growth conditions on the biomass of *P. multocida* P_{52} in fermenter.

For production of vaccine batch, working seed is prepared from the MSL (Annexure-3) and, depending on the facilities available and scale of production, subjected for cultivation by any one of the methods (Annexure 3) to produce dense culture.

9.3.2.2. Inactivation and standardization of antigenic biomass of dense culture

Dense cultures produced in the way described in Annexure-3 are inactivated by the addition of 0.5% (V/V) formalin (36-40% formaldehyde solution). Once a dense suspension of inactivated bacteria is obtained, it is standardized and adjuvanted to produce one of the 3 types of vaccine used against HS.

It is recommended that each dose (3 ml) of HS vaccines should contain not less than 2 mg of dried P_{52} (or *P. multocida*) cells (Bain *et al*., 1982). For fulfilling this requirement, dense culture harvest needs to be standardized for antigenic mass before adjuvantation.

Once a relationship between dry weight and turbidity of growth is established, turbidimeter/opacity readings can be used as an index of dry weight. It has been worked out that one Brown's Opacity corresponds to 100 mg dry mass of *P. multocida* per 1000 ml of suspension. When the opacity of the dense harvest is adjusted to 14, it contains 140 mg dry mass of *P. multocida* (P_{52}) /100 ml and both gel, and OAV vaccines, produced as per the respective formulations recommended for them, contain not less than 2.5 mg biomass per dose of 3 ml. The opacity of dense culture is therefore adjusted to Brown's scale 14 by diluting it with appropriate volume of formal saline.

9.3.2.3. Formulation of vaccine and adjuvantation

1. **Alum Precipitated Vaccine**

   To a volume of formalinized suspension of bacteria add enough of 10 per cent (w/v) (or of a hot 20 per cent w/v) potash alum to give a concentration of 1 per cent alum in the finished product. It is necessary to adjust pH of suspension to 6.5 so that flocculation occurs.

2. **Aluminum hydroxide gel adjuvant vaccine**

   To 60 parts of the standardized bacterial suspension, 40 parts of 3% (w/v) aluminium hydroxide gel is mixed. The finished product contains about 2.7 mg of antigenic mass and 36 mg of aluminium per dose of 3 ml of the vaccine.

3. **Oil adjuvant vaccine**

   To 15 parts of suspension containing desired biomass of P_{52} is emulsified with 10 and 1 parts respectively of sterilized light liquid paraffin and pure anhydrous lanolin. The finished product contains about 2.6 mg of antigenic mass per dose of 3 ml.

9.3.2.4. Filling and storage

The final batch is distributed to containers in an aseptic manner in an area not used for production**.** Containers are sealed as soon as possible with a material that is not harmful to the vaccine and that is capable of maintaining a hermetic seal for the shelf life of the vaccine (WHO, 1965). Filling operations are conducted in such a manner that eliminates chances of any contamination or alteration of the vaccine.

Vials and rubber stoppers are sterilized separately by autoclaving at 121°C for 30 minutes. Required filling equipment, sterilized vials and stoppers are transferred to the filling room pre-decontaminated by a validated process to have Grade A environment (Table 7; Annexure-1). **The processed vaccine batch is transferred into a sterile vaccine filling tank fitted with magnetic drive and is mixed slowly for 1 hour at Room temperature**. The vaccine is filled into multiple

dose vials (100 ml) using a pipetting machine transferring measured quantities of vaccine from the filling tank into the final containers**.** The vials are closed with sterilized rubber stoppers and sealed with aluminium collars immediately**.** Filled vials are labelled appropriately, kept in aluminium boxes and stored at 4°C.

It is recommended that Oil adjuvant vaccine (OAV) should be stored at 4 °C for 2 weeks prior to release because it improves the stability of the emulsion. Do not freeze OAV, as the emulsion cracks. It is also advised to keep the vials of OAV at Room Temperature at least for 1 h just before use, as this considerably improves the syrigeability of the emulsion.

9.4 Quality control of HS Vaccines:

The quality of immunobiologicals is ensured through the application of Quality Assurance (QA) System, which incorporates Good Manufacturing Practices (GMP) as one of its important component. GMP is that component of the QA system which ensures that products are "produced consistently, and controlled to the quality standards that are appropriate to their intended use"/

GMP encompasses 3 aspects production: process of manufacturing, manufacturing facilities and documentation, and incorporates Quality Control (QC) as one of its most important requirements of GMP which is concerned with sampling (during process, and of finished lots), and testing for required quality parameters through application of tests appropriate for the product.

9.4.1. Fundamental concept of Quality Control (QC) tests

Before release for sale, supply or use, each batch of vaccines/immunodiagnostic reagents/immunosera must essentially be shown to me*et al*l the required standards of quality appropriate to their intended use. The quality of vaccines and immunodiagnostic products is judged by three parameters.

1. **Safety:** The vaccine/product should not produce any undesirable effect after inoculation or exposure.
2. **Efficacy:** The vaccine/product should produce the expected effect i.e. it should induce a protective immunity in the target host/s.
3. **Quality** concerning **manufacturing and analytical standards**.

Based on these quality parameters, the QC of vaccines/immunodiagnostics essentially requires testing for their **safety**, and **efficacy** or **potency**. Additionally, as vaccines/immunodiagnostic reagents cannot be sterilized during the terminal phase of production, and sterility of the product is concerned with the safety, the products are also tested for **sterility** (in case **inactivated preparations**), and for **freedom from contamination** (in case of **live preparations**) by an appropriate *in vitro* method.

For meeting out the requirements of Good Manufacturing Practices (GMP), QC testing, not only of the final product, but also of the production process is obligatory. Process validation incorporates some additional QC tests at various stages of the manufacturing process.

9.4.2. Regulatory frame-work of quality assurance system in India:

Standards for **sterility, safety** and **efficacy** of veterinary vaccines and diagnostic reagents are established by relevant laws and directives in each country or region. In India, the quality standards of all veterinary biological products intended for distribution are regulated under the Schedule F1 of the Drugs and Cosmetic Act, 1940 and Rules 1945, and fulfilled as per the accepted testing protocols of Indian Pharmacopoeia (IP). The IP is a compilation of official standards for all drugs manufactured in India. The Eighth edition of IP was published in the year 2018 and was implemented from July 01, 2018. The part IV of the edition contains Veterinary Monographs on Veterinary Biologicals.

9.4.3. Quality control of HS vaccines in India:

The production of HS vaccines is based on **seed lot system**. A seed lot system can be considered as the back bone of GMP because it is a system in which successive batches of the vaccine are derived from the same master seed lot (MSL) of a strain which has been characterized thoroughly for its identity and purity, and tested for its safety and immunogenicity for the animal species for which the vaccine produced from it is intended for use**.** This ensures production of all vaccine batches that are consistent with respect to their quality. Before production of batches for release, it is obligatory for a manufacturer to test the vaccine seed lot for its safety and potency in target animal species. The master seed of vaccine strain is therefore subjected to extensive testing in target animal species i.e. buffalo and/or male cattle calves by the manufacturing unit and, all records thereof are kept for GMP compliance. A thoroughly characterized MSL offers an additional advantage that efficacy and safety tests on each subsequent batch of HS vaccine prepared from it can not only be limited, but may also be conducted on experimental animals like mice and rabbit. For routine production, a working seed lot (WSL) is prepared from the accepted MSL.

Three quality control tests, sterility, safety, and potency are carried out during different stages of manufacturing process, and on batches of HS vaccines. The test protocols recommended by IP (2018) are followed and described in Annexure-3.

Sterility test is carried out on culture harvests after inactivation, and on each batch of vaccine before release, for detecting absence of bacterial (both aerobic and anaerobic) and fungal contamination. Record sheet for each preparation tested/batch is maintained.

Safety and potency are very important quality parameters of every inactivated or live vaccine. The vaccine should not produce any undesirable effect and, should produce the expected effect i.e. it should be safe, and induce a protective immunity in the animal species for which the vaccine is intended to be used/the target host/s. Manufacturers are therefore required to **test the safety and potency of the HS vaccine strain in buffalo/cattle** for preparation of characterized MSL. Subsequent batches of HS vaccine prepared from a characterized MSL may then be tested on experimental animals like mice.

Potency test of HS vaccines may however, may be done on any one of the 4 animal species i.e. cattle or buffaloes, rabbits or mice (Mittal *et al*.,, 1979; Jaiswal *et al*., 1983; Johnson *et al*., 1993). Each animal species has some advantages and disadvantages, but the results on cattle or buffaloes- the target species for vaccination, are considered the most reliable. Decision on selection of animal species for potency testing of HS vaccines may depend on several factors which include feasibility, economics, availability of facilities and animals, and individual preference depending on past experience with the vaccine. Due to economic considerations it is not possible to conduct potency in cow or buffalo calves every time. Therefore the potency test on a new seed lot must be done once on any one of the target species i.e. cattle or buffaloes before accepting it for vaccine production. It is also essential to perform potency in cattle or buffalo calves on the first batch of vaccine prepared from a new MSL. All vaccine lots produced from this characterized seed lot may be tested in mice or rabbits depending on the availability of facilities (IP, 2018).

9.4.4. Shelf-life, stability and keeping quality

A well prepared OAV emulsion appears pure white, and sticks to glass, like paint. If the emulsion shows signs of cracking, it should be discarded. Separation of a thin layer of oil on the surface is permissible.

Both stability and potency are influenced by storage temperature, 4-8°C being better than room temperature (17-33°C). Vaccines can be stored at 4–8°C for 6 months without any significant loss of potency but, must not be frozen. In general, vaccines can be kept for about six months.

Increase in the content of lanolin improves stability, but also increases the viscosity (Vipulasiri *et al*., 1982; Chandrasekaran *et al*., 1987; and Gomis *et al*., 1989), which is a distinct disadvantage of OAVs/ Use of other emulsifying agents such as 'Arlacel' helps to produce thinner, stable emulsions but affects the cost of doses.

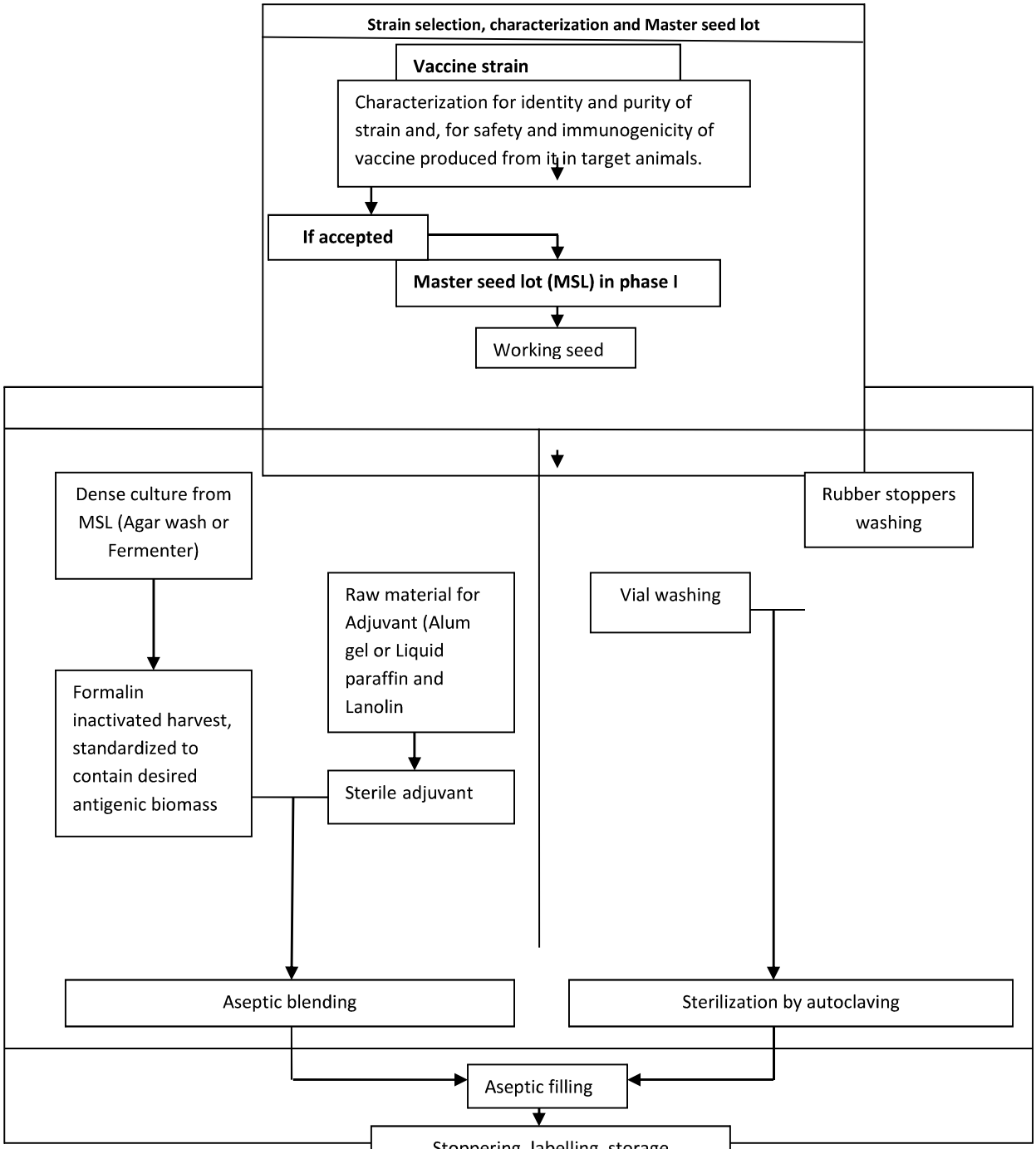

Fig. 14: Production **accines**

9.5. Vaccination

9.5.1. Rationale

Since the disease inflicts considerable economic losses to the farmers, most Asian countries, including India, rank HS as the most important bacterial disease of cattle and buffaloes. Major losses occur due to deaths in young cattle and buffalo calves; the mortality rate is almost 100 per cent in infected animals. In a recent investigation on co-occurrence of HS and FMD in Gujarat and West Bengal (Krishnamoorthy *et al*., 2017) it was found that although the prevalence rate for FMD was higher than that of HS, the mortality rate (CFR) was very high for HS in both the states. The findings clearly indicate the importance of HS in death cases and FMD in diagnosed cases. Some authors are of the opinion that farmers "fear" HS, while they view FMD and other major infections in Asia merely as "nuisance"/

HS is a bacterial disease. Theoretically, it should lend itself to effective antibiotic therapy. But treatment is constrained by a number of factors. The acute nature of most cases of the disease limits the efficacy of antimicrobial therapy of sick animals. It can be effective only if they are detected and treated in the early pyrexial phase of the disease (Chapter 8). As the disease occurs in places with substandard husbandry practices, most cases escape early detection. HS can however, be prevented by vaccination and is the pasteurellosis against which effective vaccines are available. Considering the epidemiological status of the disease, economic losses inflicted to the farmers and practical limitations of an effective therapy, vaccination appears to be the only effective control option.

9.5.2. Vaccination schedules

Depending on the type of vaccine used (APV, Gel or OAV), ease of access to animals and other practical considerations, different countries have developed different vaccination schedules. In some endemic countries yearly prophylactic vaccination is done routinely whereas, a few countries practise ring vaccination when outbreaks occur. For routine yearly vaccination programmes in endemic countries, including India, well prepared and quality tested OAV, which is thought to provide protective immunity for one year, should be used as per the following recommended schedule.

1. Carry out primary vaccination of all calves at **four to six months** of age. In farm animals, a booster dose should be given after six months. Annual vaccination should then be continued for 3 years.
2. In free-range animals that cannot be selected and restrained for vaccination, carry out annual mass vaccination of all animals **over four months of age**, **preferably two to three months before the monsoon season**. Where possible, all calves under one year of age should be re-vaccinated six months later (i.e. between two annual mass vaccinations).
3. Vaccinated pregnant dams during **six to seven months of pregnancy**. This will ensure peak antibody levels at the time of parturition and provide maximal maternal immunity to the calves.

During an outbreak, vaccination of all animals over 4 months of age should be continued. As an early protective response is desired, broth bacterins or the alum precipitated (or aluminium hydroxide gel) vaccines are preferred over OAV. However, aluminium bacterin and the oil adjuvant vaccine may be administered at different sites simultaneously. Such a schedule, if practised rigidly with coverage of over 70% of animals, will help to eliminate losses to a considerable degree.

India produces ~75 million doses of HS vaccines annually. Of these, only a small percentage is of oil adjuvant vaccine which is used only for superior stock in organized farms. Elsewhere, once yearly vaccination is practised, with mass-produced alum-precipitated or gel adjuvanted preparations just before the rainy season.

9.5.3. Adverse vaccine reactions

9.5.3.1. Post vaccination shock

Post-vaccination shock reactions, particularly with dense broth bacterins or the alum precipitated or aluminium hydroxide gel vaccines, have been reported in a small proportion of animals. The frequency of such reactions ranges from 0.1 % to as high as 10% (Bain *et al*., 1982; De Alwis 1999). Acute post-vaccinial shock response has not been observed with the oil adjuvant preparations, but injection of cracked emulsion has produced such reactions (Bain *et al*., 1982). The occurrence of reactions is irregular and therefore cannot be reproduced predictably. Affected animal generally shows symptoms of shock immediately after administration of vaccine but sometimes, symptoms appear several hours later. An acute fulminating type of response characterized by laboured breathing, sweating, in-coordination, prostration, severe colic, blood stained frothy discharges from the mouth and nostrils, followed by death is observed. In less severe or mild cases, dullness, increased respiration, frothy discharge and diarrhoea are generally observed. Post- mortem examination of fatal cases generally reveals presence pulmonary oedema, congestion of the intestines, endocardial haemorrhages and pericardial and pleural effusions. The

entire syndrome resembles one of anaphylactic shock, and is comparable with the picture observed in endotoxic shock, such as that observed in HS itself. Immediate parenteral use of antihistamines and adrenaline may sometimes be successful in saving the animal from post-vaccinial shock.

Although there are no controlled trails to establish their specific roles, some factors have been considered to have association with post-vaccination shock reactions. The age of the culture appears to be an important factor because, when very young cultures are used in vaccine production, reactions appear to be more frequent. The precipitation procedure may also be important because, when the supernatant of alum precipitated preparation is removed and bacteria are washed and resuspended in fresh formalinized saline, the occurrence of shock reactions is virtually eliminated. In addition, some soluble bacterial cell surface components (e.g. LPS and endotoxins) that are liberated into the growth medium and found in the supernatant, and other extraneous protein material present in the growth medium may also be responsible for causing the reaction.

During 2016 (Rawat, unpublished data, Division of Standardization, IVRI), 146 animals of a village in India were vaccinated with HS gel vaccine. Within one hour of vaccine administration, most animals developed symptoms of in-coordination, respiratory distress, abdominal pain with arching backs and frothy discharge from nostrils and mouth. Emergency treatment with parenteral administration of dexamethasone, chlorpheniramine and antipyretics was given, but 2 animals died. From further investigations, it was found that the gel content of some multi-dose vials of the batch, including the vial used for vaccination, was very high. This may happen due to improper filling process. In Thailand, it was once reported that of a batch of 206 animals administered with the aluminium hydroxide gel vaccine, 16 developed shock reactions (De Alwis 1989).

Some animals show local reactions and develop lumps at the site of administration. The occurrence is very rare and irregular. Nagarajan *et al*., (1975) reported that the administration of the alum precipitated vaccine results in a local reaction in some animals that gradually leads to induration resulting in a persistent hard lump. The authors compared the normal alum vaccine at a pH ranging from 5.0 to 5.4 with vaccine adjusted to pH 7. In rabbits, neutralization of the vaccine prevented local reactions. In cattle and buffaloes, it reduced the reaction but did not eliminate it altogether. Additionally, local abscesses caused by infections at the site of inoculation may also be found as post-vaccinial reaction in a small number of animals. In situations where abscesses occur frequently, it may be necessary to sterilise the skin surface of animals just before administration (this is not usually necessary unless there are visible deposits of mud or dung).

There is a belief, particularly among farmers, that vaccination of pregnant animals could result in abortions. But, De Alwis (1999) could not find any co-relation between vaccination and abortion in pregnant animals. No reports of such occurrences are available, and no systemic investigations pertaining to post-vaccinal abortions have been done in India.

KEY POINTS

1. HS vaccines contain ~ 2. 0 mg dry mass/dose of an inactivated HS-causing *P.multocida* mixed with an adjuvant.
   - In India, a highly virulent *P. multocida* strain P_{52} (B: 2) of IVRI is used for production. The strain may be obtained from ICAR-Indian Veterinary Research Institute, Izatnagar.
   - A wild strain can also be used for production, provided the strain is thoroughly characterized for identity, purity, virulence, safety, and immunogenicity in target host/s.
2. Vaccine manufacturing process involves 5 steps.
   - Selection of reference or a wild type strain and characterization for its identity by routine bacteriological methods. Maintenance of the strain in phase-1 by an appropriate method.
   - Stringent safety and immunogenicity trial in cattle / buffalo calves on experimental batches of vaccine produced from the selected strain. Preparation of master seed lot.
   - Production of dense culture.
   - Inactivation of dense culture and its standardization to contain desired biomass.
   - Adjuvantation.
3. Three types of HS vaccines are produced in India and other countries.
   - Alum Precipitated Vaccine (APV) induces a protective response for ~5 months.
   - Aluminium gel vaccine also produces a protective response for ~5 months but shows less post-vaccinial reactions as compared to APV.
   - Oil Adjuvant Vaccine (OAV) induces a protective response for about one year.
4. Choice of vaccine and schedule for immunization depend on several factors.
   - For routine annual vaccination programmes in an endemic region/country, OAV is recommended. In India, APV or Gel vaccines are used just before the rainy season.
   - For emergency immunizations during outbreaks, APV or gel vaccine is preferred.
   - The vaccination schedule should include immunization of pregnant dams, and all four-to-six month-old calves followed by a booster dose at one year and annually thereafter.
5. Up to 5 million doses a year of oil adjuvant vaccine can be made by small-scale cottage industry methods using vortex tanks. For large quantities a factory scale approach using fermenters is needed.
6. In India, quality of vaccines is regulated by applying quality control tests according to the protocol of the effective Indian Pharmacopoeia.
7. Animals may rarely show adverse post-vaccinial local reactions and fatal shock. .

10. IMMUNE RESPONSE

10.1. Naturally acquired immunity

The phenomenon of naturally acquired immunity was first described by Baldrey (1907) and Holmes (1910) and was 'rediscovered' by Bain (1954) among buffaloes in Thailand.

A small proportion of animals possess natural immunity to HS. Although, no specific data is available for India, according to Bain *et al.*, (1982), about 10% of random selections of cattle or buffaloes in Asia are naturally immune. Based on investigations of De Alwis and Sumandasa (1982) it is generally accepted that most of the naturally acquired immunity comes from subclinical infection, but some unusual findings of its incidence prove that various other factors may also be related to this phenomenon. For example, animals were found to possess naturally acquired immunity in absence of any clinical cases in Chad and Perreau (Bain *et al.*, 1982). Occasional cattle have been found in Australia with mouse protective antibodies (Bain 1954). Sawada *et al.*, (1985) and De Alwis (1999) found mouse protective antibodies among cattle with no history of exposure to HS in the United States. In these instances, it was presumed that, immunity may have been induced by related serotypes of *P. multocida*, such as B: 11in Australia, sharing common antigens.

The area specific investigations conducted in Sri Lanka by De Alwis and Sumandasa (1982) clearly indicated that incidences of naturally acquired immunity were higher in older animals and almost all the adult animals in high HS incidence areas became immune. The investigators also concluded that the proportion of naturally immune animals may vary from herd to herd in the same locality and from time to time in the same herd and is directly related to the recent incidence of HS in the locality or herd. In small groups, 50-100% animals may be found naturally immune to HS in such condition. A locality with a sizeable outbreak of HS remains free from the disease for the next year or two even if vaccination is not practised.

Although the epidemiological importance of naturally immune animals cannot be over emphasized, these animals present a practical problem during experimental immune response studies (Rawat, 1983) and potency testing of HS vaccines. Animals selected for such trials must be free from naturally occurring anti-*P. multocida* antibodies. Therefore, selection of animals should be done after vigorous screening, as failure to take account of this very common phenomenon would invalidate experiments. An ELISA-based protocol developed by El-Eragi *et al.*, (2001) and Passive Mouse Protection Test (PMPT) can be applied for detection/quantization of maternal and naturally acquired antibodies in calves. In India, an ELISA kit based on monoclonal antibody and OMP antigens of *P. multocida* has also been developed (Anonymous 2005) for evaluation of immune status of vaccinated animals, The kit can also be used for other purposes such as detection of cattle and buffaloes with naturally occurring antibodies. For the purpose of selecting sero-negative animals for any experimental trial, the rapid slide agglutination test with live *P. multocida* suspension (Annexure-3) is, however, the most practical, quick, and satisfactory method (Rawat, unpublished data on Quality Control testing of HS vaccines; Misra, 1991).

10.2. Humoral and cell-mediated responses

P. multocida is unequivocally accepted as an extracellular pathogen. A humoral immune (HI) response in concert with PMNs is efficient in controlling infections caused by extracellular pathogens and thus, protection against HS-causing *P. multocida* appears to be predominantly antibody mediated. Cell mediated immune response (CMI) appears to play only a minor role in protection, if any. Whereas, the role of humoral immunity in protection against HS has been clearly proved by passive transfer of antibodies to homologous (in serum therapy of cattle and buffaloes) and heterologous (mice: in passive mouse protection tests; *below*) hosts, protective role of sensitized T cells has never been established after their passive transfer to susceptible homologous or experimental hosts.

10.3. Immune response to HS vaccines

Though the protective response to HS vaccination has long been considered to be antibody mediated (Collins, 1977), functional immunoglobulin (Ig) classes involved in protection against natural and experimental infections, and their specificities for the antigenic components of *P. multocida* remain poorly characterized. Although the protective role of antibodies has been well established, antibody titers determined by various *in vitro* serological methods such as IHA and ELISA do not correlate with protection against challenge. Additionally, the threshold level of serum antibodies of immunized cattle and buffaloes (or of an immunoglobulin class) capable of providing 100% protection against challenge has not been determined and therefore, minimum antibody titer of a barely immune animal remains unknown. Specific information on all these aspects of immune response against HS vaccines in target hosts and experimental animals will help in developing better vaccines and diagnostics, in developing *in vitro* test systems that can preclude use of animals during potency testing and, also, in understanding the mechanism of pathogenesis.

Rawat (1983) investigated IgM, IgG-1 and IgG-2 response of cattle calves to HS oil adjuvant vaccine by using Sephadex G-200 gel filtration, Immunoelectrophoresis (IEP), Single Radial Immunodiffusion (SRID) and Tube Agglutination tests. On 7th day of vaccination, an increase in serum mg/ml concentrations of IgM and IgG-2 was observed which persisted/peaked up to 14th day, and then declined to their pre-vaccination levels. On 7th day on the other hand, levels of IgG-1 either did not show any rise, or showed a slight decline over the pre-vaccination values. The mg/ml level of IgG-1 started increasing from the 14th day up to 21st day, and then showed a declining trend. The Ig class and subclass response of could be well correlated with serum agglutinin titers which showed a rise from 7th day and peaked on 21st day of OAV vaccination. On 28th day the agglutinin titers of most calves showed a decline. The natural anti-*P.multocida* and early post-immunization agglutinin titers could be correlated with IgM class. The later titers were found associated with rise in the IgG-1 subclass of serum immunoglobulins, in general.

Samanta (2001) compared the immunoglobulin class response of rabbits to whole cell bacterin with that of purified LPS of *P. multocida* P_{52}. Whole cell bacterin induced an initial response predominated by IgM, with subsequent rise in IgG class of antibodies which were found capable of providing protection against direct challenge. On the other hand, purified LPS induced a predominant IgM response alone (with a minor rise in IgG) which failed to protect rabbits against challenge. The investigation clearly indicated that IgG, but not IgM, has functional role in protective mechanisms against HS-causing *P. multocida*. Earlier, Pati (1994) and Rawat *et al*., (2000) have also found that anti-*P.multocida* IgM does not have a role in protection. On the contrary, presence of specific or cross-reacting serum IgM against *P. multocida* may contribute in pathogenesis of infection.

In an attempt to purify and characterize the so called 'immunodominant' outer membrane protein (OMPs) or the 'most potent immunogen' of *P. multocida* (P_{52}) by Immunoaffinity Chromatography (IAC), Mishra (2006; 2009) found that when immunoglobulins from pre-challenge serum of calves that were protected against challenge, and from hyperimmune anti-P_{52} were used as ligands for binding antigens of crude lysate of the organism, 4 proteins with apparent molecular weights of 16, 33, 47, 83 kDa could be purified. Immunization of rabbits with pooled preparations of affinity purified proteins resulted only in partial protection against challenge indicating that a satisfactory protective response involves other cell wall antigens in combination with these OMPs.

In case of cattle and buffaloes, the general agreement of most investigators on the question of role of antibody with protection against challenge is that both are directly correlated. But the lowest minimum pre-challenge antibody titer for protection is still not known for both these species. This value is not only important in the context of determining the immune status of immunized or naturally immune animals, but also help in developing a dependable *in vitro* test procedure for potency testing of HS vaccines.

On the basis of analysis of available literature on all these aspects, it can be concluded that our current understanding about the induction and functions of immunoglobulin class against heterogeneous antigens of HS-causing *P. multocida* is incomplete. Whatever information is

available, relates to serological activity (Carter, 1964; Chandrasekaran *et al*., 1993, 1994a and b; Dawkins, *et al*., 1991; Giridhar, *et al*., 1990; rather than dynamics and specificities of immunoglobulin classes (Kaeberley, 1973), and their specific roles in protection, if any.

10.4. Research methodologies for conventional and novel HS vaccines

10.4.1. Animal species for immune response trials

Various vaccines used in field (Gel vaccine, oil adjuvant vaccine) and experimental vaccines (live attenuated, subunit, recombinant vaccines) are tested in target or experimental animals for their protective efficacy and duration of immunity.

1. Cattle and Buffaloes

Use of cattle or buffaloes becomes essential during several stages of vaccine development, particularly in all experiments targeted for determining dose response and duration of immunity, and in comparing different vaccines and schedules. Since HS vaccines are intended for use primarily in cattle and buffaloes, safety and potency tests on seed lot of a new vaccine candidate or reference strain are conducted on any one of these species as per regulatory requirement.

Every animal included for the experimental trial should initially be screened for absence of natural anti-*P.multocida* antibodies by an appropriate method (Annexure-3; 10.1 *above*). Control, unvaccinated animals, similarly screened, should be kept with the vaccinated groups from the beginning of the experiment to monitor any naturally acquired immunity during the progress. For the short-term experiments, at least 3 animals in the vaccinated and 2 animals in control group should be included. For longer term trials 8 or 10 animals should be used to allow for losses. For comparative trials on various types of vaccines, the test products should be prepared, where practicable, from the same bulk harvest batch of bacteria and should be standardized for same antigenic mass per dose for each vaccine. Being a useful figure of proven efficacy in several countries, the bacterial dry mass of each vaccine product to be compared should be adjusted to 2 mg/ dose.

Indirect tests for antibody response may be started at any time after vaccination.

With a group of ~20 animals, the results may be erratic in the first month, particularly is slowly absorbed adjuvant vaccines are used. A very early rise in antibody, such as on the second day after inoculation, suggest an anamnestic reaction in partial immunes. For determination of potency of vaccine batches, challenge is done on 21st day after inoculation. For immune response trials, challenging of groups may be done at any time, but it seems irrelevant before the sixth month if the vaccine under test is to be of any practical value. Animals which have given positive indirect tests for immunity are usually immune to challenge. However animals giving negative tests may also survive a reasonable challenge.

2. Mice

Mice are very susceptible to HS-causing *P.multocida* strains, and respond well to cell wall antigens of the organism when given parenterally in one or doses of 20 to 50µg. Many investigators have found a good correlation between the passive mouse protection test and immune status of cattle and buffaloes. This indicates a similarity in mechanism of immunity in the 3 species. Because of these characteristics, mice are generally used in ordinary potency testing by direct challenge after active or passive immunization.

However, the challenge tests after active immunization of mice may sometimes not be comparable to those conducted on cattle and buffaloes. A vaccine which protects mice satisfactorily may fail in cattle at the usual 2 mg dosage. Killed vaccines, for example, made from blue variants protect mice well while eliciting a poor protective response in cattle (Bain *et al*., 1982).

3. Rabbit

Although rabbits are very susceptible to HS-causing *P.multocida* strains and produce high-titre antisera against the organism after multiple inoculations, they are considered less satisfactory for use in ordinary potency testing either by direct challenge or by using their sera in mouse protection tests. Individual animals usually show a very pronounced variation in antibody responses to a single dose, and large groups are rarely available.

10.4.2. Tests for measurement of antibody response

For evaluation of protective efficacies of conventional or newly developed HS vaccines, determination of antibody titers is an essential requirement of all immunization trials where direct challenge on all immunized animals is not possible. A variety of serological tests have been used in different countries to measure immunity in vaccinated animals. These include slide agglutination test, tube agglutination tests, indirect haemagglutination (IHA), passive mouse protection test (PMPT), and enzyme-linked immunosorbent assay (ELISA). Several investigators have used PMPT, IHA and ELISA to study the duration of immunity (DOI) in vaccinated animals in controlled trials, as well as for evaluating herd immunity under natural field conditions. Some investigators have also attempted to correlate the antibody titres determined by these assays to the outcome of direct challenge in controlled experiments.

1. Indirect Haemagglutination Test (IHA)

The IHA test which was originally developed for capsular typing of *P.multocida* (Annexure-3) has also been used to detect immunity of immunized animals in research. But its reliability as an index of protection in all instances remains controversial. Sharma and Joshi (1983) used IHA test to assess the immune response in HS vaccinated and non-vaccinated cattle using LPS as antigen. They suggested that the test can be used to detect immune response of animals against vaccination and to detect possible breakdown of immunity under field conditions. On the contrary, Chandrasekaran *et al*., (1993a and b; 1994a and b) did not find any correlation between pre-challenge IHA titers and active protection against challenge. The investigators suggested that whilst the IHA test is effective in detecting antibodies produced in response to natural exposure (live organisms), its sensitivity in detecting response to HS bacterins with or without adjuvants, is poor. The reliability of IHA in evaluating antibody response to vaccination, thus needs to be reassessed.

2. ELISA

Indirect-ELISA on sera samples collected at different time intervals from vaccinated animals is a commonly used test during vaccination trials. The specificity of ELISA in detecting either antigens or antibodies depends on the nature of antigen preparation. Various antigenic preparations that include, whole bacterium, antigens heated at 56 and 100°C, sonicated whole cells, capsular and lipopolysaccharide antigens, potassium thiocyanate extract and sodium salicylate extract of *P. multocida* B: 2 have been evaluated on buffalo calves serum samples by indirect ELISA. Superiority of capsular antigens in assessing protective status of calves against HS by indirect-ELISA has been reported (Qureshi and Saxena, 2014). Earlier, the test standardized by Johnson *et al*., (1972), used a boiled antigen of B: 2. The antigen preparation contained crude LPS and the investigators could detect high titers in animals immunized with an oil adjuvant vaccine prepared from a homologous strain. Chandrasekaran *et al*., (1993a, b), comparing several serological tests and direct challenge in buffaloes, found that whilst IHA and PMPT results did not correlate with protection to direct challenge in buffaloes, there was a marked correlation between the ELISA titers and survival following challenge.

Once the test is standardized and established in the laboratory, it appears to be more suited than other tests for screening large number of serum samples. When evaluating

the antibody response to vaccination, most investigators consider ELISA to be the most appropriate test.

3. Passive Mouse Protection Test (PMPT)

PMPT was originally used for serotyping of *P.multocida* isolates. The PMPT involves inoculation of mice with sera of vaccinated cattle and challenging them with a defined dose of live, virulent organisms. Despite some limitations, PMPT is now considered a very satisfactory test for determination of 'immune statuses' of cows and buffaloes following either vaccination or natural exposure (Gupta & Sareen 1976; Chandrasekaran *et al* 1994; Myint *et al*.,, 2005). This is because; the defined lower limit of PMPT makes it a very reliable test.

Since the time of Baldrey and Holms (1907), it has been established that 20 ml of a good hyperimmune serum protects cattle and buffalo for up to one month against virulent challenge of 0.1 ml of the organism. The potency of these sera, as determined by PMPT was found to be usually one 50% Mouse Protective Dose (MPD_{50}) / 0.005 ml of serum. That means, 1 ml of a good quality hyperimmune serum contains 200 MPD_{50}. A buffalo weighing ~400 kg with a total of plasma volume of 16,000 ml, when immunized passively with 20 ml of the hyperimmune serum would receive 4000 MPD_{50}. In buffalo this would be diluted to 1MPD_{50}/4 ml of plasma. It means that only 5µl hyperimmune serum / 4 ml plasma of animal indicates a protective status of the animal against challenge.

The protocols employed by different workers vary considerably with respect to 3 variables of the test: the number of mice used for each serum sample tested, the volume of serum injected, and the challenge dose of *P.multocida*. Protocol for a routine PMPT has been described in Annexure-3.

KEY POINTS

1. A small number of animals possess natural immunity to HS and show presence of antibodies to B: 2 *P. multocida* when tested by Agglutination, IHA, ELISA and PMPT.
   - Animals selected for potency testing of HS vaccines must be free from naturally occurring anti-*P.multocida* antibodies
2. Protective response to HS vaccination is predominantly antibody mediated. Cell mediated immunity appears to play a minor role, if any.
3. A classical antibody response characterized by initial rise in IgM, followed by IgG switch over is observed following immunization.
4. Functional role of immunoglobulin classes against heterogeneous antigens of HS-causing *P.multocida* is poorly understood.
   - Pure LPS predominantly induces an IgM response which is non-protective.
   - Inactivated whole cell and protein antigen/s produce a classical IgM-IgG response.
5. Research and Development on HS vaccines.
   - Cattle or buffalo calves, mice and rabbits are used for testing immunogenicity and duration of immunity of novel vaccines.
   - IHA, ELISA and PMPT are generally employed for evaluation of antibody response of vaccines during trials.

11. AREAS FOR FUTURE RESEARCH

11.1. Host-parasite interactions and pathogenesis of *P.multocida* (B:2)

Although the sequence of patho-biological events that lead to fatal outcome of B:2 infections have been elucidated to some extent (Chapter 6), the organism still remains a pathological mystery. This is because of 2 reasons.

Firstly; it still remains uncertain that HS-causing *P.multocida* strains possess any virulence factors, and, if they do, they are merely phenotypic characters or have a genetic basis. For example, there is currently no evidence that strains of HS-causing *P.multocida* produce any exotoxin. It has been observed that serotype B: 2 strains produce hyaluronidase (Carter and Chengappa, 1980), but no role of this enzyme in causing a fatal outcome in some animal species has ever been established. Secondly; though B:2 *P.multocida* is not a specific host-adapted pathogen (like some serovars of *Leptospira* and *Salmonella*), it seems to cause fatal disease only in some animal species (Buffalo, Cattle, Rabbit and Mouse) but not in some others (Sheep, Goat, Guinea pig). The fundamental reasons as to why some animal species are extremely susceptible to natural and / or experimental infections with HS-causing serotype, and others show remarkable resistance, still remain unknown. In addition, although, Horadagoda *et al.*, (2001) have demonstrated that a massive rise in the endotoxin level of terminally infected buffaloes occurs immediately before death and, the patho-biological effects of endotoxin of HS-causing *P.multocida* have been demonstrated by producing experimental HS in calves and pigs (Rebers and Rhoades 1967), the mechanism/s involved with this massive and abrupt rise of endotoxin (LPS) level in serum of a susceptible animal species has not been understood.

Till now, there has been an **overemphasis** on phenotypic (Harper *et al.*, 2006) and genotypic bases (Hunt *et al.*, 2001; Evers *et al.*, 2006) of expression and characterization of **pathogen-associated virulence factors,** and determination of their specific roles in pathogenesis of the disease (Chapter 4). But a general review of the findings clearly indicates that the fatal outcome of B: 2 infections cannot be attributed to mere expression of those virulence factors (capsular polysaccharide and LPS). Since susceptible and resistant host animal species can be grouped on the basis of occurrence of natural disease or outcome of experimental infection, the fatal outcome of infection appears to be a result of a series of complex interactions between both the pathogen and host-associated factors. It therefore, becomes logical to visualize that **outcome of infection is a host-dependent phenomenon,** and many key factors of HS-causing strains are yet to be identified. The studies on Forssman Antigen and macrophage vacuolating activity of HS-causing *P. multocida* are very interesting and need further investigations.

11.1.1. Forssman Antigen (FA) as a putative virulence determinant

Through careful analysis of available information, it was realized in general that those **animal species which are susceptible to fatal B:2 infection do not express Forssman antigen (FA)** and are regarded as Forssman negative, whereas those animal species that show resistance to natural and experimental infection, express the antigen either on their RBCs of tissues, and are Forssman positive (Table 5; Muhlradt, 1998). *P. multocida* has been reported to be a Forssman antigen positive organism long back (Buchbinder, 1935). These observations indicate that HS-causing *P.multocida* causes fatal septic shock (HS) only in those animals species that lack FA, and thus, delineates a role for the antigen in regulating host-*P.multocida* (B: 2) compatibility.

On the basis of this background information, Singh (2014) attempted to investigate the role of FA of *P.multocida* B:2 in pathogenesis and protection of HS. He observed that whereas, FA negative animal species (cow and buffalo) posses low titers of naturally occurring anti-FA serum antibodies belonging to IgM class, FA positive animals lack these antibodies. These antibodies cross-react with FA positive, HS-causing *P.multocida*, and may play a significant role in large-scale intravascular bacteriolysis during the bacteraemic phase. This may lead to an abrupt and massive release of LPS which eventually induces a **cytokine storm** in the host, terminating in fatal shock (Chapter 6; Fig. 9).

11.1.2. Macrophage vacuolating factor/s of B: 2 *P.multocida*

It has been found that the classical Asian serotype (B:2) is capable of causing vacuolation and eventually lysis of macrophages. In a model using mouse peritoneal macrophages and, in *in vitro* investigations using a mouse macrophage cell line, macrophage lysis activity has been demonstrated with HS-causing type B strains, but not with the non-HS B strains, (Shah *et al*., 1996). The factors that mediate macrophage lysis are not characterized. But the activity is presumed to diminish phagocytic clearance and thus, promote multiplication and **haematogenous spread of bacteria**. Further investigations may lead to identification of these factors. Additionally, these studies may provide an answer to the question that why serotypes of A cause only localized lung infections (pneumonia) but not generalized shock in almost all animal species except birds?

11.2. Research on vaccine development

HS of cattle and buffaloes and FC of poultry are the only two economically important Pasteurelloses against which safe and effective immunizing agents are commercially available. HS vaccines are inactivated bacterial whole cell suspensions of an accepted immunogenic strain of *P. multocida*. These preparations are widely employed to carry out immunization programs in most countries where disease is endemic. But none of the commercially licensed formulation fulfils the criteria of an ideal vaccine because of several limitations which are generally concerned with production process, protective efficacy, stability, ease of administration, and duration of immunity (DOI).

The first, and most important of these limitations is the requirement for large amount of antigenic biomass. Being a poor immunogen, not less than 2.0 mg dry weight of inactivated *P. multocida* whole cells/dose is required for a vaccine preparation to be of any practical utility. Because of this, the production technology becomes complex and uneconomical. In countries where the disease is endemic, it practically becomes difficult to fulfill the annual demands of the doses of standard vaccines. Further, addition of one or the other adjuvant adds to the problems such as reactogenicity and post vaccination shocks (Chapter 10), viscosity and difficult administration (*below*).

The second limitation of HS (and FC) vaccines relates to their cross-protective efficacies. The conventional HS bacterins fail to induce protective response against serotypes of serogroup A and thus, have a very limited value. *P.multocida* A serotypes are important bovine naso-pharyngeal commensal, bovine pathogen and common isolates from fatal bovine and bubaline respiratory disease complex (BRD), including both enzootic calf pneumonia of young dairy calves and shipping fever of weaned, stressed beef cattle(Chapter 1). In the BRD complex, usually more than one serotypes of pasteurellae are involved as playing a role that is secondary to some respiratory viruses and stress. Likewise, Fowl cholera vaccines incorporating one or more serotype (A:1 , A:3 and/or A:4 of inactivated *P.multocida* fail to induce a heterologous protective response in poultry birds though, the birds that recover from natural infection are solidly immune to infection with any avian serotype. There are reports that although vaccination reduces the incidence of FC, the disease does occur in flocks that have been properly vaccinated even with mixed serotype vaccines. Considering the wide variety of diseases caused by the organism in various animal and bird species, and a wide spectrum of serogroups and serotypes associated with the diseases, it becomes desirable that the immunizing agent must at least be capable of imparting an across-serotype, if not an across-serogroup immunity.

Till now, there is no prophylactic coverage available against these secondary Pasteurelloses. Vaccines are considered to be of limited use, because multiplicity of etiological agents such as viruses is thought to be involved. But a prophylactic coverage against the pasteurellae involved will definitely help to reduce the severity of the pneumonic conditions, since **generally it is not the viral infection** (of any respiratory virus) **or stress, but secondary bacterial multiplication that contributes to both the severity and fatality**.

Considering all these requirements, and limitations of the conventional HS vaccines, numerous attempts have been made during the last 50 years to improve them. These attempts may

be classified into 2 broad categories depending on the basic approaches applied. Some of the studies were conducted for improving adjuvants, and the other targeted the antigenic components.

11.2.1. Improving adjuvants

A. Adjuvants other than alum, gel or oil

Several experimental vaccines incorporating adjuvants other than alum, gel, or oil were investigated. Bhatty (1973) used 2% sodium alginate, and claimed the DOI of the alginate vaccine equivalent that of oil adjuvant vaccines (OAV). In Iran, a saponin-lysed vaccine was introduced in the 1930s which was subsequently replaced by formalized-saponin vaccine (FAO, 1979), but none of the saponin vaccines gained any acceptance. Additionally, because of immunological mechanism of adjuvanticity of saponin, which has been proved to be correlated with the cholesterol content of the cell (Bangham *et al*., 1962), saponin remains an adjuvant of doubtful activity with bacterial cells, since the prokaryotic cells do not possess sterols in their cell membranes. Recently, Abdel Aziz *et al*., (2015) evaluated efficacy of Montanide ISA-70-VG oil as alternative adjuvant to the used white mineral oil with Span-80 for Fowl cholera vaccine. But besides other problems, none of the preparations containing a new adjuvant addressed the basic limitations of the requirement of high antigenic load and inability to induce cross-protective response, and proved no better than the conventional compositions.

B. Improving Oil Adjuvant Vaccine (OAV)

To address the problem of high viscosity of OAVs (water in oil; W/O), double emulsified (water-in-oil-in-water; W/O/W), preparations having low viscosity were developed and tested extensively in animals (Mittal *et al*., 1977, 1979; Yadav & Ahooja 1983; Uppal 1983; Chandrasekaran *et al*., 1993b, 1994a; Verma & Jaiswal, 1997). But production of double emulsion vaccine is more complicated than that of OAV, since only a half number of doses, as compared to W/O preparations, can be produced with the same antigenic mass. That is why, large-scale production of HS vaccines using double emulsion oil adjuvant technology never gained momentum in any country (De Alwis, 1999).

However, the external aqueous phase of W/O/W emulsion was exploited as a vehicle for incorporating an antigen other than that incorporated in the internal aqueous phase. Kumar, 2004; Kumar *et al*., 2007, compared the immune responses of 2 bivalent W/O/W preparations incorporating P_{52} in the internal and alum precipitated *P. multocida* serotype A: 1(a common cause of BRD and FC) in the external phase, and vice versa, in rabbits. The investigators observed that the antigen incorporated in the external phase induced an early and high antibody response which declined after ~28th day. In comparison, the antibody levels against the organism incorporated in the internal phase showed a delayed rise, but high titers persisted throughout the 90 day period of studies. Rabbits immunized with both bivalent vaccines showed 100% protection against both serotypes on 21st day. On 90th day however, only a partial protection was observed against the organism in external phase. The rabbit groups immunized with a monovalent P_{52} or A: 1 preparation showed protection only against homologous challenge. These observations open up possibilities for further investigations towards developing bivalent vaccines against HS which can provide protection against pneumonic pasteurelloses. Similarly, some other bacterial, viral or purified antigens may be incorporated in the external or internal phase of double emulsions to formulate combined vaccines. Rawat *et al*., (2004), incorporated tochoferol (Vitamin E) in the external phase of double emulsion HS vaccine as an immunomodulator and observed an enhanced antibody response.

11.2.2. Search for better immunogens

Numerous attempts for improving HS vaccines by identifying most potent antigen/s have also been made during the last 50 years. Experimental preparations incorporating physico-chemically extracted membrane proteins of B:2 (Ramdani and Adler, 1993; Srivastava, 1996;, Pati *et al*., 1996; Borkakowaska-Opacka & Kedrak, 2003; and Tabatabai & Zehr, 2004); OMPs expressed *in vitro* under iron-restricted conditions (Kennet *et al*., 1993; Rawat & Jaiswal, 2004); lipopolysaccharide (Joshi, 1982; Muniandy & Mukkur, 1993; Adler *et al*., 1996; Samanta and Rawat,

2005); capsular material of B and E serogroups (Penn & Negi, 1974, 1976; Giridhar *et al*., 1990; Muniandy *et al*., 1993) were studied extensively. Similarly, live, naturally attenuated (Myint *et al*., 2005) and experimentally modified and attenuated (Chengappa and Carter, 1979; Schimmel, 1993; Adler *et al*., 1996; and Hodgson *et al*., 2005) were investigated. These preparations were reported to be effective in experimental and sometimes in field conditions. But the selection of appropriate vaccine goes beyond matching of the desired and realized immune response. In general, all of them suffered from the limitations concerned with safety, protective response, DOI, and practicability of large-scale production. Although, live vaccines may provide an appropriate immune response, they are generally less preferred than non-living vaccines.

An overall critical analysis of the investigations reveals that although, the attempts at finding **most potent antigen** for an ideal HS vaccine have met with varying degree of success, none was found to possess all or some of the merits so that the improved preparation could replace the existing vaccines for routine prophylactic use. The major reasons that were associated with partial protection of the candidate immunogens could be identified however, and 2 important conclusions were drawn.

- ✓ Any single antigenic component, linked with a specifically identified gene, cannot induce total protection. No single component of *P.multocida* is entirely responsible for induction of protective immunity; all cell wall fractions including LPS, proteins and polysaccharide capsule contribute towards immunity. It is therefore unlikely that a DNA recombinant vaccine will appear within the foreseeable future.
- ✓ Monospecific immune response of inactivated vaccines relates to the alteration of antigens during the process of chemical and physical inactivation or protein extraction or at the level of antigen presentation.

Parry *et al*., (1977) found that live *E. coli* were superior to heat-killed or formalinized organisms at eliciting specific mucosal immunity. The reasons were thought to be the alterations/degradation in the antigenic moieties by chemical or physical inactivation of bacteria. Similar observations have also been made with *P. multocida*, especially the FC strains, where it has been demonstrated that the vaccines prepared from *in vivo* grown strains elicit a cross-protective response (Brogden and Rimler, 1882; Wang and Glisson, 1994; Ibrahim *et al*., 2000).

It was therefore conceived that a method of inactivation or antigen extraction that does not or minimally alter the structural configurations of antigenic moieties is the key to developing a cross-protective immunizing prepartion. A prepartion containing complete range of structurally unaltered antigenic components of *P. multocida* is not only expected to mimic a non-replicating live organism in the host and induce the desirable 'cross-protective' response at significantly low doses but also circumvent other problems of killed, sub-unit or live attenuated vaccines. For that, use of 'phage-lysed' culture of *P.multocida* was considered as an option having great potential (LeLouet, 1925).

A. Research on Bacteriophage-lysed *P.multocida* cultures as vaccines

A consistently lytic bacteriophage against a *P.multocida* type A strain was isolated, partially characterized (Durairajan, 2012) and used for generation of standardized lysates against the indicator strain of *P. multocida*. The lysate preparations were characterized for antigenic contents by SDS-PAGE and Western Blot analysis before conducting immunogenicity trials in cattle and birds. The first phase protective efficacy trials in cattle calves (Durairajan, 2012) indicated that the preparation was capable of affording protective immune response against challenge with both P_{52} and A:1 for not less than 180 days at a very low antigenic dose (0.25 ml; S/C). In birds, similarly, the preparation was found to elicit a protective response against challenge with A:1, A:3 and A:4 serotypes (Nagdive, 2017). Observations of these primary trials indicate that phage-lysate technology offers a great option through which a new class of improved immunoprophylactic agents for control of HS, FC and secondary pasteurelloses can be produced at a very low cost and, integrated with immunization programs in a more efficient manner. Further research in this area is therefore needed.

B. Research on Combined Vaccines

A combined vaccine consists of two or more antigens, either combined by the manufacturer or mixed immediately before administration, which is intended to protect either against more than one disease or against one disease caused by different strains or serotypes of the same organism. The idea of combining vaccines protective against various diseases with the objective to decrease the number of injections and, hence, to increase overall vaccine coverage, is not a new concept in vaccinology. Examples of combination vaccines in use for many years are diphtheria-tetanus-pertussis, measles-mumps-rubella, and mixtures of polysaccharide antigens to form polyvalent vaccines against pneumonia.

The use of HS-BQ and HS-FMD combined vaccines offers great advantages in countries where all the three diseases coexist. But, standardization of combined vaccines and vaccinal strains and detailed knowledge of their safety, efficacy, and potency and of the duration of immunity against each disease are needed before rational recommendations can truly be made. Only a limited number of studies have been conducted on these aspects of combined HS-BQ and HS-FMD vaccines.

A combined vaccine against HS and FMD was experimentally developed and tested in rabbits (Afzal and Muneer 1990). This water in oil emulsion vaccine gave protection to rabbits against both diseases comparable with the protection afforded by the two vaccines administered separately to rabbits.

Some immunological and efficacy trials on experimental HS-BQ vaccines prepared by different combinations have also been done. HS-causing *P.multocida* and *Cl. chauvoei* seem to be compatible antigenically, and combined vaccines protect immunized animals against challenge by either. In investigations conducted by Sinha and Prasad (1973) and Srivastawa *et al*., (1976), antibody responses against HS were assessed by IHA and PMPT. Direct challenge tests against both diseases were carried out 3 to 4 weeks (Sinha and Prasad *loc cit*), and 215 days (Srivastawa *et al*., *loc cit*) after vaccinations. The best results were obtained with the preparation that contained a suspension of *P. multocida* type B equivalent to Brown' opacity tube 16 (5 parts), aluminium chloride precipitated black quarter vaccine (10 parts), liquid paraffin (10 parts) and lanolin (1 part), mixed in a Waring blender to give a stable emulsion. Rajkumar and Saseendranath (2003) evaluated immune response to combined FMD, HS and BQ oil adjuvant vaccines in comparison to their respective monovalent vaccines in cattle. The investigators observed that all the 3 schedule of vaccination provided sufficient protective titre for FMDV type, 'O', 'A', 'C' and 'Asia-1 and *P. multocida* and *Cl. chauvoei* and concluded that the efficacy of combined vaccine was comparable to that of individual vaccines administered separately.

But, as described before, the selection of appropriate vaccine goes beyond matching of the desired and realized immune response. Requirement of the combination vaccines in different states/regions of the country (and the other countries) varies depending on the prevalence of the disease, priority status given to each disease and animal species affected by the disease. For example, black quarter is not common in areas of Asia most usually affected by HS, so that combined HS-BQ vaccine would only have limited application in those areas. Krishnamoorthi et el. (2017) mapped HS and FMD outbreaks for Gujarat and West Bengal on basis of village-level time series data (Gujarat; 2006-17) and (West Bengal; 2009-2016). The investigators found high cumulative outbreaks of both HS and FMD in both these states. So immunization by combined HS-FMD vaccine in these states will have immense advantage over the use of individual vaccine or by use of simultaneous immunization procedure with the 2 vaccines. A long term epidemiological data on each disease are therefore required before rational recommendations on use of combined vaccines can truly be made. About 6 million doses of HS-FMD and 29 million doses of HS-BQ vaccine were produced in India during 2018-19 (Tiwari *et al*., 2020).

11.3. Development of alternative and *in vitro* tests for Potency evaluation of HS vaccines

Currently, potency test on HS vaccines involves immunization with test preparation, followed by a virulent challenge of immunized and control animals. The test may be done on any

one of the 4 animal species i.e. cattle or buffaloes, rabbits or mice. Choice of animal species largely depends up on the stage of production of vaccine and the regulatory provisions of the country. The following description is based on regulatory requirements rather than on merits and demerits of the testing procedures.

It is mandatory that Master seed of a new vaccine strain or a new MSL of a conventional strain (e.g. P_{52}) is tested for potency on one of the target animal species against challenge before the start of production of commercial batches for field use. No *in vitro* or alternative test can be employed for potency evaluation of MSLs. Subsequently, according to the current regulatory requirements (Indian Pharmacopoeia, 2018), the manufacturers need to carry out a potency test on mice on every 5th batch produced from the accepted MSL. This is a cumbersome procedure that may be replaced by a reliable *in vitro* test. Immunochemical methods can substitute mandatory *in vivo* potency assays of HS vaccine batches during the production process and can help in implementing the concept of 4Rs i.e. reduction, replacement, refinement and rehabilitation (Hendriksen *et al.*, 2002). According to EC directives a potency test can be conducted up-stream of production process.

Immunochemical methods employed to detect or quantify either antigens or antibodies may be standardized but, for HS vaccines, detection or quantitative determination of *P. multocida* or its cell wall antigens appear to be a better option than quantification of antibodies produced in response to them, because the antigens can be detected or quantified at a stage up-stream of the production process, before inactivation and / or addition of adjuvant. Two main types of immunochemical methods may be utilized for quantitative determination of antigens. These include methods in which labelled antigen or antibody is used, and the methods in which unlabelled antigen or antibody is used. Mishra (2006 and 2009) detected 4 proteins (16, 33, 47, and 83kDa) of P_{52} using hyperimmune bovine anti-P_{52} serum by Immunoaffinity Chromatography (IAC) and recommended that IAC can be standardized in-house by the manufacturers, and can be accepted as an *in vitro* test for potency assay of HS vaccines up-stream of the production process on standardized bulk harvest. But this system requires costly reagents and will add to the cost of production. Therefore, Rawat (2007) standardized a modified tube agglutination test using live *P. multocida* P_{52}, pre-challenge bovine serum with known agglutinin titer from protected immunized animals, and hyperimmune bovine anti-P_{52} serum. This test can also be reliably adapted for *in vitro* potency assays by detecting antigen (*P. multocida*) on standardized bulk harvests before inactivation and can circumvent the requirement of the same in mice on finished product.

Some investigators have also attempted to correlate the antibody titres determined by IHA, ELISA and PMPT to the outcome of direct challenge in controlled experiments. But, antibody quantification methods appear to have a very limited use in potency evaluation of HS vaccines at present.

The major limitation restricting the practicability of employing an antibody quantification-based test is that, the minimum pre-challenge antibody titer for protection against challenge is still not known for cattle and buffaloes. In case of both the species, the general agreement on the question of correlation of antibody titer with protection against challenge is that both are directly correlated. But the minimum pre-challenge antibody titer for protection against challenge is still not known. The value of the threshold level of protective antibody titer is not only important from the point of view of determining immune status of naturally immune animal but also for evaluating immunogenicity (potency) of HS vaccines.

In spite of the fact that the tests involve immunization of target animals since sera samples of animals immunized with test batch of vaccine are essentially needed, they may still offer a great advantage over the conventional procedure. If standardized on MSL by establishing a correlation with protection after challenge, IHA, ELISA or PMPT may circumvent the mandatory requirement of challenge infection, and may replace it.

KEY POINTS

1. Host-parasite relationship and pathogenesis of HS-causing *P. multocida* are poorly understood. Roles Forssman antigen and macrophage vacuolating agent/s should be explored.
2. For development of a nearly ideal *P. multocida* vaccine capable of inducing an across serotype and serogroup protective response at a very low antigenic dose, use of bacteriophage lysates as vaccine provides an option with great potential.
3. Further research in to production process and protective efficacy of HS-FMD combined vaccine is required. In areas where HS and FMD co-exist, use combined HS-FMD vaccine offers some advantages.
4. Standardization of an *in vitro* test system that can be used for quantitative or qualitative determination of antigenic mass during production process will eliminate/reduce use mice for potency testing.
5. Standardization of an antibody quantification test-IHA, ELISA or PMPT in relation with protective efficacy of vaccine strain may eliminate direct challenge of control animals.

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ANNEXURE-1

Laboratory facilities, practices, and Biosafety requirements

A. GENERALLY REQUIRED CHEMICALS AND DISINFECRENTS FOR BIOSAFETY PRACTICES

A.1. Chemicals, disinfectants and disposables

1. Formaldehyde (37% v/v) solution, Potassium permanganate ($KMnO_4$), Ammonia (NH_3), Methyl alcohol, Phenol (Carbolic acid), commercially available disinfectants and hand wash from reputed suppliers.
2. Disposable bags, gloves and face-masks.

B. FACILITIES REQUIRED FOR DIAGNOSTIC BACTERIOLOY LABORATORY

P. multocida is a biohazard Risk Group 2 pathogen (OIE, 2008). Routine diagnostic procedures **do not involve production of aerosols or splashes**. Therefore, clinical specimens and suspected or confirmed isolates of the organism are handled in a Biosafety Level 2 laboratory with modest facilities.

B.1. Infrastructural facilities:

Biosafety Level 2 (BSL 2) containment facilities are required for operations involving cultivation of bacteria in small quantities. The laboratory should be easy to clean. Its surfaces should be impervious to water and chemicals. Facility of wash basin, eye bath and emergency shower should be available in lab. Entry to laboratory should be restricted.

B.2. Equipment

1. Compound **binocular microscope** with 10x, 45x and 100x objectives, and mechanical stage
2. Laboratory **Autoclave** with automatic temperature time control, and pressure gauge
3. Laboratory **Hot Air Oven** with automatic temperature and time control, and temperature display
4. Laboratory **Incubator**
5. Bench top centrifuge machine
6. Water bath
7. Class-I microbiological safety cabinet
   (i) A class I microbiological safety cabinet (can be operated in open-front mode) should be used. A class II cabinet provides less operator protection but may be used only when- handling large quantities of cultures and generating aerosols.
8. **Bunsen Burners** with Gas Supply
9. **Platinum/Nichrome wires and wire holders**
10. UV lamps
11. Single glass distil water unit
12. Refrigerator and deep freeze (-20°C)

B.3. Glassware and plasticware

1. Microscopic slides and cover slips

2. Glass Petri dishes (90 mm diameter) and Pre-sterilized plastic Petri dish (individually packed)
3. Glass test tubes of different size and capacities
4. Pre-necked glass ampoules for freeze drying of ~0.5-1ml culture suspension
5. Pre-sterilized plastic centrifuge tubes of 15 ml and 50 ml capacities
6. Test tube racks
7.

B.4. Media and chemicals

1. Agar agar powder
2. Aluminium chloride
3. Ammonium oxalate
4. Andrade's Indicator
5. Blood agar Base
6. Brain Heart Infusion broth and agar Base
7. Crystal violet/Gentian violet
8. Dextrose/Glucose
9. Dipotassium hydrogen phosphate (K2HPO4)
10. Di-sodium hydrogen phosphate (Na2HPO4. 12H2O)
11. Ethyl alcohol
12. Ethylene diamine tetra-acetic acid (EDTA)
13. Formaldehyde (37% solution)
14. Giemsa's stain
15. Sterile, inactivated horse serum
16. Iodine
17. Kovac's reagent
18. Meat (Beef) extract
19. Methyl alcohol
20. Nutrient broth Base
21. Oxidase reagent or Tetramethyl-p-phenylene-diamine dihydrochloride
22. Pancreatic digest of Casein
23. Papain digest of Soybean Meal
24. Peptone
25. Peptone water Base
26. Potassium Iodide
27. Potassium dihydrogen phosphate (KH2PO4)
28. Rectified spirit
29. Resazurin sodium
30. Safranin
31. Skimmed milk powder
32. Sodium chloride
33. Sodium dihydrogen phosphate (NaH2PO4. 2H2O)
34. Sodium glutamate
35. Sucrose

36. Tryptic digest of casein
37. Tryptose phosphate broth base
38. Xylene
39. Yeast Extract

B.5. Other consumables

C. FACILITIES REQUIRED FOR MOLECULAR BIOLOGY WORK

C.1. Equipment

The following are essentially required for molecular biology laboratory:

- ✓ Centrifuge (microfuge and bench-top centrifuge)
- ✓ Water bath(s) (standard temperatures 37°C, 50°C and 65°C)
- ✓ Pipettes (ranging in volume from 0.1 µl to 1 ml)
- ✓ Pipette tips
- ✓ Plastic tubes (0.2µl or 0.6µl PCR tubes, 1.5-ml Eppendorf tubes, 35-ml and/or 50-ml centrifuge tubes)
- ✓ Submerged Gel Nucleic Acid Electrophoresis System
- ✓ Power Pack 300 (or 200) system
- ✓ UV transilluminator and dark room facilities
- ✓ Photographic films (Polaroid 31/ 4 x 41/ 4 inches)
- ✓ Polaroid camera
- ✓ Vortex

C.2. Special equipment

- ✓ Thermal cycler
- ✓ Pipettes (preferably two complete sets, pre- and post-PCR handling)
- ✓ Microfuge tubes (PCR tubes of 0.2 or 0.6 ml depending on the thermal cycler specifications; and 1.5 ml tubes)

C.3. Consumables

- ✓ Culture media
  - (i) Agar agar powder
  - (ii) Blood agar base
  - (iii) Brain Heart Infusion broth base (BHI)
  - (iv) Tryptone soya broth base (TSB)
- ✓ **PCR analysis**
  - (i) Thermostable DNA polymerase (Taq, Tth, Pfu, Pwo etc.)
  - (ii) PCR buffer
  - (iii) Deoxynucleotide triphosphate set (dATP, dCTP, dGTP and dTIP)
  - (iv) Oligonucleotide primers (see individual assays for specific sequences)
  - (v) Magnesium chloride
  - (vi) Nuclease-free water
  - (vii) Light mineral oil (see thermal cycler specifications)

(viii) Agarose for electrophoresis

(ix) Tris-acetic acid-EDTA (TAE) buffer

(x) Ethidium bromide

(xi) Loading dye

(xii) DNA markers (either Bacteriophage lambda cut with EcoRI and Hindlll or 100 bp DNA) (the marker should be adequate for all DNA analysis purposes).

C.5 Details of buffers and solutions required are shown in Annexure-2.

D. FACILITIES REQUIRED FOR VACCINE PRODUCTION AND QUALITY CONTROL

For vaccine production **in general**, compliance of good manufacturing practices (GMP) requires an environment that integrates (i) Product protection (ii) Operators safety, and (iii) Environment protection**.** These are conflicting requirements and need careful adjustments that may be done by: air filtration, air pressure differentials or physical segregation of activities. **Training operators play the most important part in fulfilling these requirements.** *P. multocida* has been placed under Risk Group 2 by the OIE. Also, with long experience of production of HS vaccines in India (which are inactivated and adjuvanted suspensions of bacteria), it can be concluded that a vaccine strain is **not such a potential bio-hazard organism** for which specific and costly environment protection measures like air filtration systems and air pressure differentials are absolute requirements.

The aspect of environment protection cannot be overemphasized in case HS vaccine production facilities. Probably, due to this understanding, initial workers thought of developing a technology capable of producing up to 5 million doses of HS Vaccine annually at small-scale, **"Cottage Industry"** level.

The other 2 aspects viz. product protection and operator's safety require more attention during HSV production. These can be, to a large extent, taken care by operators training and discipline only. The availability of qualified and experienced scientific and technical manpower is more critical than anything else in the entire process of HS Vaccine production.

HS vaccines can be produced under the **Campaign system** of production which allows culture of more than one organism within a manufacturing area, provided the activities are segregated in time and that a validated decontaminating procedure has been followed between each cycle of activities. Whatever the case may be the bacterial population in the environment of the work areas of HS Vaccine production must be kept within permissible limits. Though, this may be achieved by providing filtered air supply and laminar flow cabinets, the entire system is costly, difficult to maintain and becomes inappropriate for those small-scale production units that largely fulfill the demands of poor farmers of their area. It is therefore necessary that **small-scale State Biological Production Units should adopt such practices that circumvent need of the costly air handling systems**. In absence of filtered air supply systems, a validated decontamination process, such as regular fumigation (Annexure-1; E) of laboratories, work cabinets and areas should be followed and the level of air-borne organisms should be regularly monitored to keep them within the permissible limits (Table: 9)**.**

The following stages of HSV production require "clean areas" of specified grades for product protection and operator safety:

1. **Cultivation and Harvest:** Grade A environment with a Grade B/C background
2. **Downstream processing (adjuvantation):** should be done in Grade A environment with Grade B background.
3. **Filling and Sealing:** Grade A with Grade B background

Table 9. **Recommended limits for particulate and microbiological counts of "Clean !reas" (in operation)**

Grades	Particles/m^3		Organisms			
	0.5µm	5 µm	Air sample cfu/m^3	Settled plates (90mm dia.) cfu/2hrs	Contact plates (55 mm dia.) cfu/plate	Glove points (5 fingers) cfu/glove
A	3500	0	<1	<1	<1	<1
B	3500	0	10	5	5	5
C	350k	2000	100	50	25	-
D	350k	20k	500	100	50	-

E. ROUTINE DECONTAMINATION OF WORK AREA

1. **Fumigation:** Formalin is commonly used for the purpose. It works best at temperatures above 20 °C and humidity above 65%. Depending on the area of work space, one of the three methods of formalin fumigation can be effectively employed.

   (i) **Formalin-Potassium permanganate mixture** (Misra, 1991)**:** This method is satisfactory for routine decontamination of bacterial diagnostic and vaccine testing labs where only small amounts of sporulated and non-spring bacterial cultures are handled without generation of splashes and aerosols. For 1 cubic meter space, a Petri dish containing 13 ml of 37% formalin and 6.5 g of $KMnO_4$ is placed and the area is sealed. Following overnight fumigation, the area can be used for work after removing excess formalin by exhaust fan.

   (ii) **Formalin Spray:** Moisten thoroughly all surfaces of wall, floor and furniture of the properly sealed room with a spray of 10 % formaldehyde solution (1 volume of formalin diluted with 3 volumes of water) and then saturate the atmosphere by spraying undiluted formalin to the extent of 1 liter per 1000 ft^3**.** The operator should wear an efficient anti-gas respirator during the procedure. The room is kept closed for 24 h by sealing the door. Introduce a container with Ammonia solution (1 liter ammonia solution mixed with 1 liter of water per liter of 40% formaldehyde used) into the room and leave for several hours. The room can be used for work after removing excess ammonia by exhaust fan.

   (iii) **Formaldehyde vapours:** Heat the properly sealed room to about 24°C and boil formalin (500 ml of 40% formalin diluted with 1 liter water for **1000 ft^3 space**) in an electric boiler having a safety device which kicks out when the vessel boils dry and a time switch that cut off the current. The room is kept closed for 4-24 h by sealing the door. Introduce a cloth soaked in ammonia solution (250 ml per

liter of formalin used) and leave it for 2-3 hours. The room can be used for work after removing excess ammonia by exhaust fan.

2. **Ultraviolet radiations:** This may be employed for disinfection of internal surfaces of safety cabinets. UV radiation is equally effective against Gram-positive and Gram-negative cells but spores are 10 times and viruses are 200 times more resistant. Keep the surfaces of the cabinets as clean as possible. Use a properly shielded UV source to prevent injury to the eyes and skin of the staff in the vicinity. Switch-off the lamp when the cabinet is in use. Keep the UV lamp clean and check its bactericidal activity regularly since it deteriorates with time/ (UV lamps have limited 'working life')/Check the air borne microbiological load of the **working area** and **cabinets** at regular intervals and maintain a record of them.

F. GENERAL GUIDELINES FOR LABORATORY DISCIPLINE

1. Do not allow to enter visitors and persons not directly concerned with the functions of the laboratory.
2. Do not allow any person not trained in microbiological techniques to handle cultures.
3. Use tight-weave, non-shedding protective clothing and face masks while handling the bacterial cultures and infected materials. Sterilize the used clothing material after completion of work.
4. Handle all cultures and infected materials in a suitable bio-safety cabinet or in front of flame.
5. Clean the laboratory daily before and after the work.
6. Discard infected materials, culture plates, tubes, pipettes and other contaminated materials into leak-proof disposable bags/containers and incinerate or decontaminate by autoclaving. No infected material should be discarded in sinks or any other drain. Material for disposal should be transported carefully without spillage.
7. Soak reusable glassware into a disinfectant and autoclave before cleaning. Used glassware and other material must be stored safely before autoclaving.
8. No mouth pipetting.
9. No eating, drinking or smoking in the laboratory. Food and drinks should not be stored or consumed in labs. Application of cosmetics and smoking is prohibited.
10. In the event of spillage or contamination, disinfectants must be available for thorough cleaning. Contaminated protective clothing must be removed and autoclaved. Hands must be thoroughly washed immediately. All surfaces to be de-contaminated as and when needed.
11. Care must be taken to minimize aerosol generation.
12. In case of accidents, a record should be maintained and should be reported to safety officer.

ANNEXURE-2

Preparation of media, reagents, buffers and antisera

A. TRANSPORT MEDIA FOR SUBMISSION OF CLINICAL SPECIMENS

Any one of the following transport medium may be used for dispatch of clinical specimens:

A.1. Stuart's modified transport medium

Sodium glycerophosphate:	10.0 g
Sodium thioglycollate:	0.5 g
Cysteine hydrochloride:	0.5 g
Calcium chloride:	0.1 g
Methylene blue:	0.001 g
Agar:	5.0 g
Distilled water:	1 litre
pH:	7.4 ± 0.2

All ingredients are dissolved in 1 litre of distilled water. Approximately 6-7 ml of the medium is then dispensed into screw-capped bottles of appropriate capacity and sterilised at 121°C for 15 minutes.

A.2. Transport enrichment medium (TEM) (Warner, 1996)

Agar agar:	3.0 g
BHI broth base:	37.0 g
Distilled water:	1 litre

Mix well, autoclave to sterilize, cool to approximately 56°C. Add following antibiotics from stock solutions to attain final concentration mentioned against each antibiotic.

Amikacin:	10µg/ml
Gentamicin:	10µg/ml
Potassium tellurite:	0.4µg/ml
Bacitracin:	6.0µg/ml
Amphotericin B solution:	4.0µg/ml

Dispense in 10 ml aliquots (in glass McCartney bottles or alternative screw-capped bottles of appropriate capacity). Store at 4°C.

Clinical material may be inoculated in bottles containing transport medium the in the following manner:

Heart blood, oedema fluid etc: Add fluid material to the TEM bottle and shake to mix into the sloppy agar.

Swabs: Push swab down into agar and break off into the bottle.

Tissues (Spleen, liver, lung etc): Small amounts of tissue should be pushed below the agar surface (should be less than 10% of the volume of the media);

For culturing, a sterile swab is pushed down into the agar of transport medium tube, and used to make the primary area on blood agar plates.

B. GENERAL PURPOSE AND *P. multocida* GROWTH MEDIA

B.1. Nutrient broth

Meat extract:	5 g	(0.5%)
Peptone:	10 g	(1.0%)
Sodium chloride:	5 g	(0.5%)
Water:	1000 ml	

Place weighed quantity of each ingredient in a flask of suitable size and dissolve in desired volume of water, by boiling (if needed). Adjust pH to 7.4; distribute about 5-10 ml broth in tubes of appropriate size. Cotton plug, mark, pack and autoclave tubes at 121 ° C for 20 minutes. Check sterility of the tubes by placing them in incubator for 48 h.

Alternatively, commonly available dehydrated commercial formulations are satisfactory and the medium is prepared by simply following the instruction of the manufacturer.

Sterile broth medium is not stored at 4°C for more than 15 days.

B.2. Nutrient agar

Prepare Nutrient broth in desired quantity as described and add 2-2.5% agar after adjusting pH to 7.4. Autoclave the medium by usual procedure and dispense in pre-sterilized test tubes or Petri dishes. Check sterility of each tube and plate by usual method and store at 4°C.

B.3. Brain Heart Infusion (BHI)

Commonly available dehydrated commercial preparations of BHI media are satisfactory and can be prepared by simply following the instruction of the manufacturer.

B.4. Blood agar:

It is a multipurpose medium employed for primary isolation of *P. multocida* from clinical specimens. Cultures of the organism can be routinely maintained on blood agar for all procedures. The medium is used in form of plates and slants.

Prepare desired quantity of either Nutrient agar, Blood agar base, Brain Heart Infusion (BHI) or Tryptose agar or Casein-Sucrose-Yeast Extract agar (CSY) base, according to the procedures described, autoclave, and cool to about 48°C. Add sterile, defibrinated sheep blood (10% V/V) aseptically. Distribute in pre-sterilized Petri dishes and/ or test tubes. Check sterility by incubation for 48 h and store at 4°C.

Commercially available Brain Heart Infusion agar (BHI) or Blood agar base may also be used for preparation of Blood agar.

B.5. Tryptose broth

This is another general purpose medium. It may be used for preparation of inoculums:

Tryptose:	20.0 g (2%)
Sodium chloride:	5.0 g (0.5%)
Dextrose:	1.0 g (0.1%)
Water:	1000 ml

Dissolve the ingredients in appropriate volume of water by boiling. Adjust pH to 7.4, distribute in Erlenmeyer flasks/tubes, and sterilize at 121°C for 20 minutes in autoclave.

B.6. Tryptose agar

This is a good medium for observing colonial morphology and fluorescence and, is used for identity tests of *P. multocida*. The composition given here is used in the University of Sydney.

Sodium chloride:	5.0 g
Agar:	25.0 g
Water:	500 ml

Dissolve the ingredients in about 500 ml of water by heating. Add:

Tryptose:	20.0g
Tryptone:	2.0 g
Sucrose:	1.0 g
Yeast extract:	1.0 g
Distilled water:	500 ml

Adjust pH to 7.4-7.6. Filter, autoclave and dispense in desired glassware.

B.7. Selective medium for isolation of *P. multocida*

Prepare semisolid Tryptose-tryptone base with 1% agar. Distribute in tubes in 10ml lots and sterilize by autoclaving at 120°C for 20 minutes. Before use, melt the basal medium and add, from stock solutions, the following to give the required concentrations:

Neomycin:	2.5 µg/ml
Potassium tellurite:	2.5 µg/ml
Tyrothricin:	10 µg/ml
Actidione:	100 µg/ml

Actidione may be omitted if fungal contamination is not expected.

C. MEDIA FOR BIOCHEMICAL TESTS FOR CHARCTERIZATION OF *P. multocida*

C.1. Sugar fermentation tests: Peptone water sugars

Prepare peptone water medium for each of 14 sugars- arabinose, dulcitol, fructose, galactose, glucose, innulin, inositol, lactose, mannose, raffinose, rhamnose, salicin, sucrose,

trehalose and xylose. Use a commercially available product of Peptone water or prepare according to the formulation given below:

Peptone:	3.0 g (1%)
NaCl:	1.5 g (0.5%)
Distilled water:	300 ml
pH:	7.2-7.3
Andrade's Indicator (1% v/v):	3.0 ml

Dissolve the ingredients in 300 ml distilled water, adjust pH to 7.2-7.3 and add Andrade's indicator/ Dispense ~20 ml in 14 (1 for each sugar) McCartney tubes or screw-capped bottles and sterilize by autoclaving. Add 1ml of filter sterilized 50% (W/V) solution of test sugar aseptically. Dispense in 5 ml amounts, in cotton-plugged and pre-sterilized 150 x 12.5 mm test tubes each containing a 50x7mm Durham tube. Check sterility of each tube by incubation at 37°C for 3 days.

C.2. Peptone water for Indole test:

Indole test is an important test for confirmation of identity of *P. multocida* isolates. All strains of produce indole. Commonly available dehydrated commercial formulations are satisfactory and the medium can be prepared by simply following the instruction of the manufacturer. Alternatively, the medium can be prepared in the laboratories by dispensing good quality dehydrated ingredients as per the following composition and procedure.

Peptone	2.0 %
NaCl	0.5%
pH	7.4

Dissolve the ingredients in desired volume of distilled water, adjust pH to 7.4; dispense in 5 ml amounts in cotton wool plugged 150x12.5 mm test tubes and sterilize in autoclave. Check sterility of each tube by incubation at 37°C for 3 days.

Kovac's reagent: Kovac's reagent is required for Indole test (Annexure-2). A commercially available preparation can be used with satisfactory results. Alternatively, the indicator may be prepared in the laboratories.

Amyl or isoamyl alcohol:	150 ml
p-dimethyl-aminobenzaldehyde:	10.0 g
Concentrated hydrochloric acid (HCl):	50.0 ml

Dissolve the aldehyde in alcohol and slowly add the acid. Prepare in small quantities and store in refrigerator.

C.3. McConkey's agar

Growth on McConkey's agar is an important test for identification of *P. multocida* because the test *differentiates P. multocida* strains from *Pasteurella* (*Mannheimia*) *haemolytica*. All *P. multocida* strains fail to grow on McConkey's agar even after prolonged incubation. Dehydrated commercial preparations are satisfactory and the medium can be prepared by simply following the

instruction of the manufacturer. Alternatively, the medium is prepared in the laboratory by dispensing good quality dehydrated ingredients as per the following composition and procedure:

Peptone:	2.0%
Sodium taurocholate:	0.5%
Agar powder:	2.5%
Neutral red solution (2% in 50% ethanol):	0.4%
Lactose	1.0%
pH:	7.5

Dissolve weighed quantities (according to requirement) of peptone and taurocholate (bile salt) in water by heating. Adjust pH to 7.5; add lactose, neutral red and agar. Sterilize by autoclaving and pour plates. Check sterility by incubation at 37°C for 48 h.

D. SOLID MEDIA FOR PRODUCTION OF BIOMASS FOR VACCINES IN ROUX FLASKS

D.1. Yeast extract peptone agar medium

A medium with the following composition has been satisfactorily used by the Biological Products Division, Indian Veterinary Research Institute, Izatnagar for obtaining dense cultures of P_{52}.

Beef (meat) extract:	10g	(1%)
Peptone:	10g	(1%)
NaCl:	5.0g	(0.5%)
Yeast extract:	5.0g	(0.5%)
Creatinine:	1.0g	(0.1%)
Agar powder:	30-40g	(3-4%)
pH:	7.4	

All ingredients of medium are dissolved in water with appropriate amount of heating. The medium is adjusted to pH 7.4, distributed into Roux flasks (~120 ml per flask of 1500 ml capacity); sterilized by autoclaving (121°C; 45 minutes) and cooled in horizontal position. After solidification of agar, the water of condensation is pipetted out aseptically and the flasks are incubated for at least 24 h to allow them to dry and for sterility check. It is important to have a dry agar surface before inoculation. Discard flasks showing contamination. Roux flasks are used for production of vaccine immediately.

D.2. Casein-sucrose-yeast extract agar (CSY):

This formulation is recommended by Misra (1991)

Casein hydrolysate:	3.0g
Sucrose:	3.0g
NaCl:	5.0g
Yeast extract	5.0g
Dipotassium hydrogen phosphate:	3.0g
Agar powder:	25-30g
Distilled water:	1000ml
pH:	7.4

Place weighed quantity of each ingredient in a flask of suitable size and dissolve in 1000 ml volume of distilled water, by boiling (if needed). Adjust pH to 7.4; distribute in Roux

flasks or desired glassware. Sterilize by autoclaving at 121°C for 30 minutes. Check sterility of flasks by incubation for 48 h as described above.

This medium may also be used for preparation of Blood agar.

E. BROTH MEDIA FOR PRODUCTION OF BIOMASS FOR VACCINES IN FERMENTER

E.1. Casein-sucrose-yeast extract broth (CSY):

This CSY broth with added Autodigest of Pancreas is recommended by Misra (1991). Prepare CSY broth as per the composition given above.

For preparation of Autodigest of Pancreas, remove fat and connective tissue from about 5 kg weighed quantity of pancreas collected from pig or sheep or buffalo or cattle and mince in a blender. Measure volume of the minced pancreas, add half of the volume of tap water to it and adjust pH to 9 by 10N NaOH. Keep the material in a water bath at 45°C for 5-6 hours for complete digestion. An automatic stirrer is used for better digestion. Check pH at every 30 minutes during digestion and re-adjust as it falls. Remove from the water bath, adjust pH to 4.0 by HCl and filter through muslin cloth. Boil the filtrate in boiling water for 5 minutes, cool and store overnight at 4 °C. Heat the digest to 80°C and filter through filter paper. Finally, adjust ph to 7.4, boil in a water bath for 5 minutes, filter sterilize and store at 4°C. The autodigest is best used fresh as sterile filtrate.

Good results are obtained by adding 1 litre of digest to 24 liters of CSY

F. MEDIA USED FOR STERILITY TESTING OF HS VACCINES (Indian Pharmacopoeia, 2018; Section 2.2.1)

F.1. Fluid thioglycollate medium (FTM)

FTM, also referred to as Medium 1**,** is used to test sterility, since it supports growth of both aerobic and anaerobic organisms without necessity of sealing the tubes. Dehydrated commercial products of FTM are satisfactory and convenient. However, the medium can be prepared in laboratory.

Pancreatic digest of casein:	15.0 g
Yeast extract (Water soluble)	5.0 g
Glucose	5.0 g
NaCl	2.5 g
L-Cystine	0.5 g
0.1% Resazurin Sodium Sol. (freshly prepared)	1.0 ml
Agar (bacteriological)	0.75 g
Purified water	1000 ml
Polysorbate 80 (Tween 80)*	10.0 ml

Mix the pancreatic digest of casein, yeast extract, glucose, sodium chloride, L-cystine, agar and water in the proportions specified above and heat until dissolved. Dissolve the sodium thioglycollate in the solution. Add the specified quantity of Polysorbate 80 (Tween 80) if this ingredient is to be included. Adjust pH by adding sufficient 1N NaOH or 1 N HCl so that after the solution is sterilized, its pH will be 7.1± 0.2. If the solution is not clear, heat to boiling but do not boil, and filter while hot through moistened filter paper. Add the 1 ml of 0.1%resazurin sodium solution in 1000 ml medium and mix. Commercial dried powdered preparations of FTM are also available. Simply observe the instructions provided by the manufacturer of the dehydrated mixture to prepare a clear solution of the specified pH.

Immediately prior to adjusting the pH, add the specified quantity of Polysorbate 80 if this ingredient is to be included.

Distribute the medium into clear colourless glass vessels with external screw threaded necks in the required volumes. The required volume to be filled in vessels varies according to the volume of vaccine in containers to be tested.

Medium should be sterilized within four hours of the start of its preparation. Sterilize the vessels of media by autoclaving at 121°C for 20 minutes.

Prepared medium may be stored at 2-25°C in suitable sealed containers but must not be used after storage periods that have not been validated by in-house testing. If stored for longer durations, they should be tested by growth promotion every three months. FTM, of which more than the upper one-half is pink in colour, should not be used in tests for sterility. Vessels of this medium which have become excessively pink may be heated once only in a steam bath, or in freely flowing steam, until the pink colour disappears

F.2. Soybean-casein digest medium (SCDM)

SCDM, also referred to as Medium 2**,** is required for checking fungal contamination at various stages of production and on final lot of vaccines. Dehydrated commercial products are very satisfactory. However, the medium can be prepared in laboratory as per the following composition.

Pancreatic digest of casein:	17.0 g
Papain digest of soybean meal:	5.0 g
Glucose:	2.5 g
NaCl:	5.0 g
Di-potassium hydrogen phosphate (K_2HPO_4):	2.5 g
Purified water	1000 ml
Polysorbate 80 (Tween 80)*:	10.0 ml

Mix the ingredients, in the proportions specified in above table, warming slightly to effect solution. Cool the solution to room temperature. Add the specified quantity of Polysorbate 80 if this ingredient is to be included. If necessary, add sufficient 1 N NaOH or 1N HCL so that after the solution is sterilized its pH will be 7.3± 0.2. If the solution is not clear, filter through moistened filter paper. Alternatively, follow the instructions provided by the manufacturer to obtain a clear solution of the specified pH. Just prior to adjusting the pH, add the specified quantity of Polysorbate 80, if this ingredient is to be included.

Dispense and store SCDM as described above for FTM.

G. STABILIZERS FOR LYPOHILIZATION

G.1. Sucrose-Horse serum Stabilizer

For lyophilization of most bacterial strains, sucrose-horse serum is preferred over the other cryoprotecting stabilizers by the Type Culture Lab, Division of Standardization, ICAR-IVRI. The stabilizer has been consistently found satisfactory for maintaining vaccine (and challenge) strain of *P. multocida* in freeze-dried state. The isolates freeze-dried with this cryo-protecting medium have shown to undergo minimum variations with respective to their virulence and antigenic characters during their long-term maintenance (Personal unpublished data). The following composition of the stabilizer is used.

30 % (W/V) sucrose solution (Sterilized):	1 part
Sterile inactivated horse serum:	4 parts

Harvest bacteria in sucrose solution aseptically and add sterile inactivated horse serum in correct proportion to make a thick, even suspension. Dispense in pre-sterilized ampoules and freeze dry.

G.2. Sucrose-Glutamate-Dextran (SGD) stabilizer

This medium may also be used for lyophilization of *P. multocida* strains.

Sucrose:	5.0 g
Sodium glutamate:	1.0 g
Dextran:	5.0 g
NSS	100 ml

Sterilize the SGD stabilizer by filtration. Suspend bacteria and freeze dry.

H. STAINS

H.1. Gram's stain:

Ready to use kits for Gram's stain are available commercially and can be used/However, stocks may be prepared in the laboratory and used routinely.

1. Stock crystal violet (SCV)

(i) Solution A (Crystal violet)

Crystal violet:	10.0. g
Water:	100 ml

(ii) Solution B (Ammonium oxalate)

Ammonium oxalate:	1.0 g
Water:	100 ml

Prepare working crystal violet solution by mixing 20 ml of SCV (A) with 80 ml of oxalate solution (B).

2. Gram's iodide solution

Iodine crystals:	1.0 g
Potassium iodide:	2.0 g

Dissolve iodine and iodide completely in 10 ml distilled water, and then add distilled water to make 200 ml.

3. Decolorizer

Ethyl alcohol (95%):	50 ml
Acetone:	50 ml

4. Counter stain (Stock Safranin solution)

Safranin:	2.5 g
Ethyl alcohol (95%):	100 ml

Prepare Safranin working solution by preparing 1:4 dilution of stock Safranin solution.

H.2. Giemsa's Stain

Ready-to-use preparations of Giemsa stain are available commercially. However, stock may be prepared in the laboratory and used after dilution.

Giemsa powder:	0.3 g
Glycerin:	25 ml
Methyl alcohol (Acetone-free):	25 ml

Grind Giemsa powder with glycerin in a mortar and add methyl alcohol. If stain does not go into complete solution, filter it and store in tightly stoppared reagent bottle.

I. MISCELLENEOUS SOLUTIONS AND REAGENTS

I.1. !ndrade's Indicator

Andrade's indicator is added to peptone water sugars for detecting change in pH due to acid production. The indicator becomes dark reddish-pink when the pH falls to about 5.5. A commercially available preparation can be used with satisfactory results. Alternatively, the indicator may be prepared in the laboratories by adding 1N NaOH to a 0.5% solution of Acid Fuchsin until the colour just becomes yellow.

I.2. Buffer for Agar gel precipitation test (Somatic typing)

Sodium di-hydrogen phosphate (NaH_2PO_4. $2H_2O$):	0.41 g
Di-sodium hydrogen phosphate (Na_2HPO_4. $12H_2O$):	1.46 g
Sodium chloride (NaCl):	8.50 g
Double glass distilled water:	100 ml

I.3. Neutral red Indicator

This indicator is used in McConkey's agar/It becomes rose-pink when the pH of the medium becomes acidic. At alkaline pH the colour of the indicator is yellow. A commercially available preparation can be used with satisfactory results. Alternatively, a 2% solution of Neutral red in 50% ethanol may be prepared just before use.

I.4. Normal Saline (0.15M NaCl; NSS) and Formal saline

Normal saline solution (NSS) is required various purposes. Prepare sufficient quantity of normal saline by dissolving sodium chloride in water.

Sodium chloride:	8.5 g
Distilled water	1000 ml

Distribute NSS in containers of appropriate capacity and sterilize by autoclaving, if needed.

Formal saline (0.5% or 0.3%) may be prepared by adding 5 ml or 3 ml of commercially available formaldehyde solution (37-40%) to 1 liter of NSS.

I.5. Oxidase Reagent

A commercially available preparation can be used with satisfactory results. Alternatively, a freshly prepared 1% aqueous solution of tetramethyl-*p*-phenylene-diamine dihydrochloride may be used.

J. ANTISERA

J.1. Anti-*P.multocida* rabbit serum for Rapid Slide Agglutination test on blood agar cultures (Misra, 1991)

Obtain growth of an HS-causing B: 2 strain/reference B: 2 strain or P_{52}.

Seed a young broth culture (6-8 hour) of the strain on 6 CSY agar plates. Incubate at 37°C for 18 hours. Wash off the bacterial growth with about 4 ml formal saline (0.3% V/V)/plate and check the purity of each suspension by wet and stained film examinations. Pool pure suspensions and centrifuge for 2 minutes at 1000 rpm to remove coarse particles. Collect the supernatant bacterial suspension and centrifuge at 4000 rpm for 10 minutes. Discard supernatant. Re-suspend the pelleted cells in sufficient 0. 3% (V/V) formal saline so as to match with Brown's scale 7. The suspension may be stored at 4°C for use.

Immunize 2 to 3 healthy, adult rabbits shown to be negative for presence of anti-*P. multocida* antibodies by following the schedule of Table 9.

Table 10. Immunization schedule for rabbits

Dose (ml) (i.v)	Day of injection
0.2	0
0.5	4
1.0	8
1.5	12
2.0	16

Inject 0.5 ml live *P. multocida* suspension matched to Brown's opacity scale 7 on 7th day of last injection through subcutaneous route. Bleed the animals 10 days after the last immunizing dose. Separate serum and store in small aliquots at -20°C or at 4°C after addition of Merthiolate (1:10,000).

J.2. Hyperimmune rabbit antisera for Capsular typing

Obtain growth of respective reference strain as 0.3% formalinized suspension adjusted to Brown's opacity scale 7, and immunize 2 to 3 healthy adult rabbits according to the schedule described above in section J.1. Bleed animals to collect antiserum. Store in small aliquots at -20°C or, at 4°C after addition of Merthiolate (1:10,000).

J.3. Hyperimmune chicken antisera for Somatic typing (Heddleston *et al*., 1972)

The 16 reference strains representing the 16 somatic types are used. Obtain growth of the each reference strain to prepare antigen for raising antiserum in chicken.

Seed a young broth culture (6-8 hour) of the strain on several dextrose starch agar (DSA) plates. Incubate at 37°C for 18-24 hours. Wash off the bacterial growth of each plate with about 3 ml of 0.3% (v/v) formalinized phosphate buffer saline (pH 7). Check the purity of each suspension. Pool pure suspensions and centrifuge for 2 minutes at 1000 rpm to remove coarse particles. Collect the supernatant bacterial suspension and centrifuge at 4000 rpm for 10 minutes. Discard supernatant. Re-suspend the pelleted cells in sufficient formalinized PBS so as to be equivalent to Brown's opacity standard 6/Emulsify the suspension in equal quantity of Freund's Incomplete Adjuvant (FIA)/ Immunize 2 to 3 healthy chicken aging between 12-16 weeks by a subcutaneous injection of 1 ml antigen into the neck. Three weeks later, a further 1 ml, divided into 0.5 ml volumes, is injected on each side of sternum. Exsanguinated birds 1 week later; collect sera and preserve in small aliquots at -20°C or, at 4°C after addition of Merthiolate (1:10,000). Each serum is tested against the 16 types, any that cross-react are discarded. It is

best to immunize 3-4 birds per serotype, and to collect the serum from each bird separately until cross-reacting antisera are eliminated.

K. REAGENTS FOR ELISA

K.1. Coating (Carbonate-bicarbonate) buffer

Sodium bicarbonate:	0.84 g
Sodium carbonate:	0.356 g
Double distilled water add up to:	100 ml

Store at 4°C. Use within 7 days

K.2. Blocking solution

Skim milk powder:	1.2 g
PBST:	24 ml

Prepare fresh for use.

K.3. Washing buffer

1x PBS pH 7.4:	1000 ml
Tween 20:	500µl

Store at room temperature until use.

K.4. Stock substrate solution A

Citric acid:	2.10 g
Double distilled water add up to:	100 ml

Store at 4°C until use.

K.5. Stock substrate solution B

Sodium citrate:	2.94 k
Double distilled water add up to:	100 ml

K.6. Stock working solution

Stock substrate solution A:	25. 5 ml
Stock substrate solution B:	24.5 ml

Store at 4°C until use.

K.7. OPD

Working substrate buffer:	12.0 ml
OPD:	6.0 mg
H_2O_2 (30%):	5.0 µl

Always prepared freshly and used.

L. BUFFERS AND REAGENTS FOR PCR ASSAYS

L.1. Agarose gel Electrophoresis

1. Tris Acetate EDTA (TAE) Buffer (10x), pH 8.8

Tris base:	48.2 g
Glacial acetic acid:	11.4 ml

EDTA (0.5M, pH 8): 10.0 ml

Add distilled water to make final volume of 1000 ml.

2. Tris Borate EDTA (TBE) Buffer (10x), pH 8.3

Tris base:	109.0 g
Boric acid:	55.0 g
EDTA:	9.3 g

Add distilled water to make final volume of 1000 ml

3. Ethidium bromide solution

Ethidium bromide:	10 mg
Autoclaved distilled water:	1 ml

Dissolve Ethidium bromide in water by vortexing. Store at 4°C protected from light by aluminum foil.

ANNEXURE-3

Methods, Procedures and Protocols

A. DIAGNOSTIC AND INVESTIGATIVE PROCEDURES

A.1. Mouse inoculation for isolation from contaminated specimens

Inject 2 healthy adult mice subcutaneously with 0.1 to 0.2 ml of blood, or a saline suspension of tissue (bone marrow or spleen) sample. If HS-causing *P. multocida* types are present (even in a very few numbers), both the animals will die within 24 hours.

Carry out post mortem as early as possible after death. The heart blood films and spleen impression smears of the infected mice will show abundance of bipolar staining coccobacilli when stained with Giemsa and Grams methods (*below*; Fig. 1). For isolation, streak heart blood across several blood agar plates and incubate at 37°C for 24 hours.

A.2. Microscopy and staining

1. Tissue impression or blood films

Fix blood films or tissue impression smears in methyl alcohol for 3 minutes. Prepare working solution of Giemsa's stain by mixing 1 part of stock (Annexure-2) solution in 10 parts buffer solution, pH 7.0. Stain fixed films in diluted stain for 1 hour and wash with buffer solution, allowing the preparation to differentiate for about 30 seconds. Blot dry, and observe under 100x objective to check presence of pure organisms having staining characters and morphology indistinguishable from *P. multocida.*

2. Smear from cultures

Prepare smears from agar plate or slopes- heat fix and stain by Gram's method. Observe under 100x objective to check presence of pure organisms having staining characters and morphology indistinguishable from *P. multocida* (Fig. 3).

A.3. Rapid slide agglutination test on blood agar cultures

Place a drop of NSS on a clean, grease-free glass slide. With the help of a platinum loop, mix a single colony of the isolate in the saline drop. Add a drop of anti-*P.multocida* serum (Annexure-2), mix with a match-stick and observe in oblique, transmitted light. Coarse floccular aggregate indicates a positive reaction (Fig. 14). Positive slide agglutination test on isolate supplements other tests of identity confirmation.

A.4. Preservation and maintenance of pure cultures

1. For long-term preservation

Freeze dry growth of pure isolate directly from an accepted plate/slant (in Phase-I); Use horse serum stabilizer as per the procedure described *below* (B.1; 1).

Alternatively, freeze dry defibrinated heart blood in ampoules.

2. For short-term maintenance

Streak single colony of isolate from an accepted plate on 2-3 blood agar slopes/slants and incubate. Check purity and morphological identity by microscopic examination of each slant. Mark slants with an appropriate identity number, seal and store at 4°C. On blood agar slants preserved at temperature between 4°C-8°C, *P. multocida* cultures remain viable for about 10 days. The slant cultures can be used in further tests, if needed.

A.4. Identification by conventional biochemical tests

1. Catalase and Oxidase tests

Streak 3 plates of Tryptose agar (Annexure-2) with the organism and incubate for 24 hours.

For catalase test, place a drop of Catalase reagent (Annexure-2) on a clean, grease-free glass slide and with the help of a platinum loop, mix a single colony of the isolate from a plate in the reagent drop. Development of air bubbles within 10 to 15 seconds indicates a positive catalase reaction.

For oxidase test, flood the 3 plates with a freshly prepared 1% solution of tetramethyl-*p*-phenylene-diamine dihydrochloride (Annexure-2) so as to cover the surface growth and decant in an aseptic solution. The colonies will rapidly develop a purple colour.

2. Motility

Prepare wet film of a 6 hour nutrient or BHI broth growth of the organism and observe under 40x objective. *P. multocida* is always non motile.

3. Indole production

Inoculate peptone water (Annexure-1) and incubate for 48 hours at 37°C. Add a few drops of Xylene to the culture, shake and allow the Xylene layer to settle over the medium for a few minutes. Slowly add Kovac's reagent (Annexure-2). The test is positive if a red colour develops in the Xylene ring.

4. Growth on McConkey's agar

Streak each isolate across 3 plates of McConkey's agar (Annexure-2) and incubate for 4-5 days. No growth of the organism will be obtained.

5. Sugar fermentation profile

Seed each sugar medium (Annexure-2) with a loop-full of pure culture on solid medium or a drop or loop-full of pure broth culture and incubate at 37°C. Examine the media for presence of colour change (to reddish-pink) after 24 and 48 hours of incubation.

A.5. Serological tests

1. Indirect Haemagglutination (IHA) test for capsular typing

(i) Preparation of antigen

Obtain growth of isolate as 0.3% formalinized suspension and adjust to Brown's opacity scale 6 as described in Annexure 2; J.1. Heat the cell

suspension at 56°C/60°C for 30 minutes in a water bath with intermittent shaking. Centrifuge suspension at 8000 rpm for 15 minutes at 4°C and collect clear supernatant. Store at -20°C till used.

(ii) Preparation of RBCs

Collect sheep blood aseptically by jugular puncture into a sterile flask containing suitable volume of Alsever's solution (Annexure-2), and keep at 4°C for 7days. Centrifuge a suitable volume of preserved sheep blood at 1500 rpm for 10 minutes and wash the packed RBCs three times with 5-6 volumes of chilled NSS by centrifugation. Prepare a 10% (v/v) suspension of sRBCs after the final wash.

(iii) Glutaraldehyde fixation of RBCs

Prepare a 1% (w/v) solution of glutaraldehyde in sterile NSS and chill it at 4°C. Mix equal volumes of chilled glutaraldehyde solution and 10% RBC suspension in an Erlenmeyer flask of suitable capacity and allow standing at 4°C for 30 minutes with intermittent gentle shaking. Pack fixed RBCs by centrifugation at 1500 for 10 minutes and wash 3 times with chilled NSS. Prepare a 10% (v/v) suspension of sRBCs in NSS after the final wash.

(iv) Sensitization of RBCs

Mix one volume of fixed RBC suspension and 15 volumes of undiluted antigen and incubate for 30 minutes at 37°C in a water bath with frequent gentle shaking. Wash RBCs 3 times with NSS by centrifugation at 2000 rpm for 5 minutes. Prepare a 1% (v/v) suspension of sensitized RBCs in NSS after the final wash. Store at 4°C.

(v) Adsorption of antiserum

Keep the antiserum (Annexure-2; J.2) at 56°C for 1 hour in a water bath for inactivation. Add 3 volumes of antiserum to 1 volume of packed unsensitized RBCs and incubate at 37°C for 2 hours in a water bath with periodic shaking. Remove RBCs by centrifugation.

(vi) Test proper

Carry out test on 96 well round-bottom microtiter plates.

Dispense 160µl NSS and 40µl antiserum (inactivated and adsorbed) to the first well of a horizontal row giving a 1/5 dilution of the antiserum. Dispense 100µl of NSS to all other wells. Make 2 fold serial dilutions of antiserum by transferring and mixing 100µl of mixture up to the penultimate well from which 100µl is discarded after mixing. Leave the last well of the row with only NSS to serve as serum control. Add equal volume of sensitized 1% RBCs to each well. Simultaneously, add 100µl of 1% suspension of unsensitized RBCs to a duplicate row of similarly diluted serum. This serves as antigen control. Shake the plate gently and leave at room temperature for 2 hours. Coarse agglutination of RBCs forming a matt which is distributed round the well indicates a positive haemagglutination whereas, formation of a small button of deposited cells is considered as a negative reaction (Fig.--).

2. Indirect Haemagglutination (IHA) test for determination of immune response (Sawada *et al.*, 1982)

(i) Preparation of antigen (Sonicated antigen)

Obtain approximately 5 ml growth of pure *P. multocida* as a suspension in Carbonate-bicarbonate buffer (pH 9.6) in a centrifuge tube and adjust to Brown's opacity scale 6 (~ $5x10^9$ organisms/ml) as described in Annexure 2; J.1. Sonicate the bacterial suspension 20 times at amplitude of 20 with 10 second pulse and 10 second pause on ice to lyse the cells completely. Centrifuge the lysed suspension at 8000 rpm for 15 minutes at 4°C and collect clear supernatant. Store at -20°C till used.

(ii) Preparation glutaraldehyde fixed RBCs (GA-sRBCs)

Prepare and fix sheep RBCs by following the steps (ii) and (iii), as described above (A.5.1).

(iii) Tannic acid fixation of GA-sRBCs

Prepare a 0.005% (w/v) solution of tannic acid in sterile Phosphate buffer saline (PBS; Annexure-2) and chill it at 4°C. Mix equal volumes of chilled solution and 10% GA-sRBCs suspension in an Erlenmeyer flask of suitable capacity and allow standing at 4°C for 30 minutes with intermittent gentle shaking. Pack sRBCs by centrifugation at 1500 for 10 minutes and wash 3 times with chilled NSS to remove free tannic acid. Prepare a 10% (v/v) suspension of tannic acid treated T-GA-sRBCs in NSS after the final wash.

(iv) Determination of antigen dilution for sensitization of T-GA-sRBCs

Carryout a series of IHA tests using T-GA-sRBCs sensitized with 2 fold dilutions of reference antigen prepared at step (i), using 1 volume of packed cells and 15 volumes of dilutions of antigen. The dilution of antigen beyond which the HA titer starts dropping is considered as the end point. Fix antigen dilution at 4 fold dilutions of the end point. For example, if HA titer starts dropping beyond 1 in 64 dilution then, the dilution of antigen used for sensitization of T-GA-sRBCs will be 1 in 16.

(v) Sensitization of T-GA-sRBCs for test proper

Mix one volume of packed T-GA-sRBCs suspension and 4 volumes of the dilution of antigen determined at step (iv), and incubate for 30 minutes at 37°C in a water bath with frequent gentle shaking. Wash sensitized RBCs 3 times with NSS by centrifugation at 2000 rpm for 5 minutes. Prepare a 0.5 % (v/v) suspension of sensitized RBCs in NSS after the final wash. Store at 4°C.

(vi) Absorption of sera samples

Follow step (v) as described *above*.

(vii) Test Proper

Follow step (vi) as described *above*.

3. Agar gel precipitation test for somatic typing (Heddleston *et al*., 1972)

Prepare 100 ml of 1 percent (w/v) molten Noble agar (or an equivalent) in the AGPT buffer (Annexure-2; I.2). Pour approximately 12 ml volumes of the molten agar on clean glass slides (57 mm x 70 mm) using a pipette. Keep slides at 4°C for 1 hour to allow agar to set and solidify.

When set firmly, using a punch, cut three patterns of seven (consisting of 1 central and 6 peripheral) 4 mm diameter wells each, spaced at 6 mm center to center.

Place the unknown test antigen (prepared by the method described in Annexure-2; J.3) in each central well. Five antisera (Annexure-2; J.3) are then placed in five peripheral wells, with saline or negative serum control in the remaining well. Incubate slides overnight in a warm and humid chamber.

All Indian (Asian) HS isolates will show a precipitin line with antiserum against type 2. Some cross-reactions with type 5 may also be found (De Alwis, 1999).

4. ELISA for rapid identification (HS-antigen ELISA) (Dawkins, *et al*., 1990)

The protocol developed by Dawkins and co-workers is recommended for standardization of HS-antigen ELISA in labs.

(i) Preparation of crude immunoglobulins

A heat-stable antigen of *P. multocida* isolate 0019 (Insein) (Bain 1955) was used in the original method described by the investigators. This isolate was selected for immunization as it was known to cause HS consistently, after experimental challenge of hosts. The strain was frequently passaged through cattle. Indian P_{52} strain is also known to cause HS in both cattle and buffaloes consistently after challenge. It has been regularly passaged through cattle during potency testing of HS vaccines and is maintained in phase-I in the Type Culture Lab of IVRI, Izatnagar (Rawat, 2004; Verma and Rawat, 2004). P_{52} may also be used as an antigen for immunization in place of Insein.

- **Antigen and immunization:** Prepare boiled antigen of the selected *P. multocida* strain by harvesting the growth of young culture as per the procedure described in Annexure-2; J.3, and boiling it at 100°C for 1 h in a water bath. Immunize rabbits as described in Annexure-2; J.1 to collect anti-*P.multocida* serum.
- **PEG precipitation of serum immunoglobulins:** Dilute sufficient quantity of antiserum 1:1 with PBS and make to a 13 % w/v solution of polyethylene glycol (PEG) 6000 (BDH Chemicals) by the drop-wise addition of a stock 50% w/v PEG solution. Mix the slurry for 30 minutes and collect the precipitate by centrifugation. Dissolve the pellet in PBS to a volume of the original serum plus PBS volume. Repeat the PEG precipitation. Re-suspend the final pellet in PBS and dialyse extensively against the same buffer.
- **HRP-conjugation:**

Dilute the PEG precipitated immunoglobulin fraction to 8 mg/ml in PBS. To 4 ml of immunoglobulin, add 800 µl of sodium carbonate/sodium bicarbonate solution (30 ml of 0.1 M sodium carbonate and 7 ml of 0.1 M sodium bicarbonate, pH 9.5; Annexure-2)

and stir. Working to a formula of 1 mg of horseradish peroxidase (HRP; Boerhinger Mannheim) per 2 mg of protein, dissolve 16 mg of HRP in 4 ml of distilled water, and add 800 µl of 0.1 M sodium-*m*-periodate to the dissolved horseradish peroxidase. Run the horseradish peroxidase solution through a 20-ml Sephadex G-25 column pre-equilibrated to pH 4.4 in 1 mM sodium acetate, and collect the yellow-brown fraction. Add the horseradish peroxidase fraction to the immunoglobulin solution and gently stir for 2 hours at room temperature. Dialyze the conjugated immunoglobulin preparation extensively against PBS at 4°C.

(ii) Test proper

All steps are performed at room temperature with 1OO µl volumes. Coat the polystyrene microtest plates with PBS containing 2 µg/ml rabbit anti-*P.multocida* P_{52} Ig fraction (*above*) and allow standing overnight.

- Cultivate *P. multocida* isolates to be tested, on sheep blood agar overnight at 37°C and harvest approximately 20 µg (20 µl) weight each into a microfuge tube containing 1 ml PBS. This produces a cell suspension estimated to contain more than 10^{10} organisms per ml, which is adopted as the cell concentration for positive control stock suspensions. If the bacteria are to be stored for any length of time, add formalin to give a final concentration of 0.04 % formaldehyde.
- Dilute harvested bacteria 1:10 in PBST. Wash the antibody-coated plates three times with PBST. Add the diluted bacteria to the appropriate column giving about 10^8 bacteria per well. Add each isolate to one column of the microtiter plate, giving octuplets for each sample in order to analyse the variability between wells and provide accurate standardization of the test. Also include substrate, conjugate and positive control columns. Incubate the plate at room temperature for 1 h; then wash the plate three times in PBST.
- Add conjugated immunoglobulin preparation (*above*) at an optimal dilution (1:1000) and incubate for 1 h. Wash the plates four times with PBST and add a substrate solution of 1 mM freshly prepared ABTS in 0.1 M citrate buffer pH 4.2 containing 2.5 mM H_20_2. Allow colour to develop for 1 h and read the plate with an ELISA reader using a 414 nm wavelength.

Express the results as ELISA units (OD x 100).

5. ELISA for quantification of immune response (HS-antibody ELISA)

(i) Immunization and anti- *P.multocida* serum

Obtain growth of the selected *P. multocida* strain and immunize rabbits as described in Annexure-2 J.1. Bleed the animals 10 days after the last immunizing dose. Separate serum and store in small aliquots at -20°C or at 4°C.

(ii) Test antigen

Various antigen preparations may be used in ELISAs for the detection of antibody response to HS-causing *P.multocida*. If the identity of the vaccinal strain is known, and the antibody response is to be

detected against the homologous strain, the boiled antigen is suitable for an ELISA.

Prepare boiled antigen of the selected *P. multocida* strain by harvesting the growth of young culture as per the procedure described Annexure-2 J.3, and boiling it at 100°C for 1 h. Depending on the objective of the trial, other preparation may be used as test antigen.

- Formalin (0.3 %)-killed antigen of intact cells of *P. multocida* can be prepared by harvesting young growth in 0.1 M sodium carbonate buffer (pH 9.6), and adjusting to an absorbance of 0.25 at 600 nm.
- Boiled intact *P. multocida* cell antigen can be prepared by adjusting boiled suspension to 1 x10^8 colony forming units (CFU)/ ml.
- Purified LPS antigen (10µg / ml) may be prepared by the hot-phenol method (Westphal and Jann 1965).

(iii) ELISA proper

The following protocol may be used.

- **Coating of antigen:** Coat the wells of a flat-bottom polystyrene microtiter plate (flat-bottom microtiter plates are preferred, but U-bottom plates may also be used) with 50 µl of appropriate *P.multocida* antigen, usually at a dilution of 1:300 in PBS. Either cover or place in a moist chamber and hold at 4°C overnight. Wash the plate three times (for 5 minutes each) with PBST. To reduce background, add 200 µl of either 1 % bovine serum albumin (BSA) at 4°C for 30-60 minutes or 0.1 % gelatin in sodium carbonate buffer for 2 hours at 25°C to each well.
- **Antibody:** Add 100 µl of test serum (bovine or bubaline) diluted 1:200 in PBST20 to each well. Keep control wells with no antigen, no conjugate, no serum, no substrate, and both positive and negative control sera on each plate. Incubate for 1 h at 37°C. Wash the plate as above with PBST.
- **Conjugate:** Add 50 µl of developing antiserum (HRP-labelled anti-bovine/bubaline IgG) per well, diluted to required working strength in PBST. The developing antiserum may be produced in-house in sheep using an established protocol and conjugating to horseradish peroxidase, Sigma type VI. Alternatively, a commercial preparation may be used. Wash the plate three times (for 5 minutes each) with PBST.
- **Developing:** Add 100 µl of freshly prepared substrate consisting of 0.1 M citrate/phosphate buffer pH 4.2 containing 1 mM ABTS with 2.5 mM H_2O_2 to each well. Incubate at 37°C and read after 30 minutes at 415 nm using a ELISA Plate Reader. Calculate the titer of the test serum.

- **Titers:** ELISA titer is assigned as the reciprocal of the highest dilution of serum that gives an absorbance at 414 nm (OD_{414}) of 0.50.

6. Passive mouse protection test (PMPT)

The following protocol may be routinely used:

(i) Select healthy, adult weighing not less than 20 g, mice from a colony hitherto unexposed to pasteurella and free from pasteurella antibodies for test. Use 5 mice for each serum sample to be tested.

(ii) Passive immunization:

- Serum samples of the animals under investigation are injected into each of 5 mice, divided in separate groups for each sample. Inject each of 5 mice of a group with 0.5 ml of filter-sterilized serum from an immunized animal through subcutaneous route.

(iii) Challenge

(iv) Twenty four hours after the serum injection, challenge each passively immunized mouse of each group with 100LD_{50} of *P.multocida*.

- Where the LD_{50} for mice is 1-10 colony forming units (CFU), so the normal challenge dose is estimated to contain 100 CFU. This is usually achieved by using 1 ml of a 10^{-5} or 10^{-6} dilution of a 6-8-hour CSY broth culture. As accuracy is not crucial at this stage, a visual judgment can be made to obtain to obtain a dose of approximately 100 LD_{50}.

(v) **Interpretation**

- Most workers have accepted that survival of at least one of a group of five injected mice (i.e 20% protection) as an index of protection in cattle, provided all of a similar group of control mice die.

A.6. PCR-based test

1. Template DNA

Commercially available kits that allow rapid extraction of PCR-ready DNA from various tissue types and blood may be used by following the protocol provided by the manufacturer. Alternatively, crude DNA for PCR assays may prepared by the following protocols.

(i) Crude DNA preparation from tissue and blood samples

Inoculate the swab/blood/tissue into 2 ml of TSB (Annexure-2) and grow on a roller/shaker for about 1-2 hours (or until there is visible growth). Transfer 1 ml growth into a 1.5 ml Eppendorf tube and, centrifuge at 13,000 rpm for 4 minutes. Discard the supernatant, wash in 500 µl of distilled water and centrifuge at 13,000 rpm for 4 minutes. Discard the supernatant, re-suspend in 100 µl of distilled water. Transfer the 100µl suspension to a PCR tube and overlay with mineral oil.

Boil suspension in a PCR machine at 98°C for 20-30 minutes. Centrifuge (place the closed PCR tube in a new Eppendorf tube) at 13,000 rpm for 2 minutes. Transfer the supernatant to a sterile Eppendorf tube and store at -20°C until required. Use 5 µl of supernatant in the PCR reaction.

(ii) **Crude DNA preparation from pure cultures** (Ewers *et al.*, 2006)

Centrifuge ~ 1.5 ml of overnight culture of *P. multocida* in a sterilized 2 ml capacity Eppendorf tube at 10,000 rpm for 15 minutes. Discard supernatant and mix the pellet in 200 µl NSS by vortexing. Place the suspension in boiling water for 10 minutes and snap-chill in ice for 5 minutes. Repeat the step 3 times. Centrifuge at 10,000 rpm for 30 minutes, collect the supernatant in another Eppendorf tube, and store at -20°C.

(iii) Crude DNA preparation from bacterial colonies

Suspend one to two colonies in 100 µl double distilled water and centrifuge at 13, 000 rpm for 2 minutes at room temperature. Re-suspend the pellet in 100 µl of sterile distilled water and boil at 100°C for 15 minutes. Centrifuge at 13 000 rpm for 2 minutes at room temperature. Transfer the supernatant containing the DNA to a sterile vial and keep at 4°C for routine use or -20°C for long-term storage.

2. **HS-PCR Assay** (Townsend *et al.*, 1998, 2001)

(i) The details of primers used are given in Table 9. The reaction mixture and the conditions of PCR have been given in Tables 10 and 11 respectively.

(ii) Visualize the amplified DNA products by electrophoresing of 5µl of the product in a 1% agarose gel with Ethidium bromide (0.5µg/ml)

Table 11. PCR primers for specific diagnosis of HS (HS-specific PCR (B: 2)

Primer name	Sequence of Primer	Product size (bp)
KTSP 61	5'-TATTTAGGTGACACTATAG-3'	560
KTT 72	5'– AGGCTCGTTTGGATTATGAAAG–3'	
HS-B-2-F	5'-CGAAAGAAACCCAAGGCG-3'	320
HS-B-2-R	5'-ACAATCGAATAACCGTGAGAC-3'	

Table 12. Reaction mixture for HS-PCR

S. No.	PCR reaction	Quantity
1	10x PCR buffer	2.5 µl
2	dNTPs (10 mM)	0.5 µl (200 µM)
3	$MgCl_2$ (25mM)	1.5 µl (1.5 mM)
4	Primer (F)	0.5 µl (10 pmol)
5	Primer	0.5 µl (10 pmol)
6	Nuclease-free water	16 µl
7	Taq polymerase	0.5 µl (1.5 U)
8	Template DNAs	0.3 µl (50 ng)

Table 13. Conditions for amplification of HS-specific PCR

PCR	Stage	Temperature (°C)	Time (min).	Cycle
HS-PCR	Denaturation	95	4	1
		95	1	30
	Annealing	55	1	-
	Extension	72	1	-
	Final extension	72	9	1

A.7. Hyaluronidase production: Non serological test for identification of HS-causing type B *P.multocida*

HS-causing type B *P.multocida* strains produce hyaluronidase enzyme which hydrolyzes hyaluronic acid. The production enzyme can be tested by growing the organism in vicinity of an organism that produces hyaluronic acid capsule.

1. Streak across a plate of dextrose starch agar a culture of hyaluronic acid-producing organism. Conventionally, cultures of *Streptococcus equi* have been used for the test. *P.multocida* serogroup A, which produce hyaluronic acid capsules with mucoid colonies, may also be used.
2. The pasteurella cultures to be tested are then streaked across the plate at right angles to the original streak, and incubated at 37°C for 18 hours.
3. At the point of intersection, where the test *P.multocida* culture produces hyaluronidase, the hyaluronic acid capsule producing culture will show a thinned-out growth due to decapsulation. The reading is facilitated by the use of freshly prepared plates and a humidified incubation.

B. VACCINE PRODUCTION

B.1. Seed lots

1. Preparation of Master Seed Lot (MSL)

Open a vacuum-tested, freeze-dried ampoule of the seed strain procured from the reference laboratory or, of the candidate vaccine strain, and reconstitute the contents in about 0.5 ml sterile saline. Streak the reconstituted material across 2-3 CSY blood agar plates. Incubate at 37°C for 18 to 24 hours. Check for purity and identity of growth obtained on plates and inoculate representative single colony into nutrient or BHI/Tryptose broth tubes. Incubate at 37°C for 12-18 hours. Check purity of the broth culture by microscopic examination. Prepare a 1:10 dilution of the broth culture using sterile NSS.

Inject a buffalo calf or a male cattle calf aging not less than 8 months, and testing negative to presence of natural anti-*P.multocida* serum antibodies in slide agglutination test (Annexure-3; *below*), with 1 ml of the diluted culture through subcutaneous route.

Heart-bleed the animal **aseptically** when moribund or immediately after death. Collect blood in a **pre-sterilized** bottle of suitable size containing glass beads. Slowly shake the bottle to defibrinate blood. Inoculate the infected heart blood on several blood agar slants and incubate tubes for 18-24 hours. Check for purity and morphological identity of growth on each slant. Wash the growth of accepted slants by adding about 1 ml of 30% (w/v) sterile sucrose solution (Annexure-2) to each tube. Add 3 ml sterile, inactivated horse serum (available commercially or produced in-house) to each tube and shake gently to make a homogenous suspension. Dispense 0.5 ml in sufficient number of pre-sterilized ampoules and freeze dry.

Prior to its further use for production of vaccine, characterize the freeze-dried MSL by subjecting it to tests for purity and identity. Keep all record the MSL. Maintain the freeze-dried seed lot at -20°C in both the production and QC labs.

2. Preparation of Working seed (WSL)

Use one vacuum tested freeze dried ampoule of the MSL and follow all the steps to collect infected heart blood from a challenged buffalo or male cow calf.

Store the infected blood in 1 ml aliquots in sealed cryo-tubes or ampoules at -20°C or lower. The frozen infected blood can be used for sufficient period of time, if only one freeze-thaw cycle is allowed.

Follow the steps to obtain growth on blood agar plates. Confirm purity and identity of growth and inoculate representative single colony into Tryptose broth (Annexure-2) in Erlenmeyer flasks. Incubate at 37°C for 12-18 hours. Check purity of the broth cultures by microscopic examination and if required, by streaking on fresh blood agar plates. Select pure broth cultures as inoculums. Maintain record of WSL.

B.2. Dense cultures

1. Agar wash growth

Inoculate each of the production Roux flasks, containing sufficient amount of Yeast Extract Peptone Agar medium (Annexure-2; 120-150 ml/flask) (or a suitable medium developed in-house), with 2-3 ml of seed culture. Spread the inoculum evenly on to the **completely dried agar surface**. Incubate flasks in an inverted position at 37°C for 18-24 hour. Inspect growth of each flask visually for the colony characteristics. Discard flasks showing contamination, if any. Pour-off aseptically and, discard residue of inoculum in a container with disinfectant solution**.**

Introduce about 10 ml formal saline (Annexure-2) and a few sterile glass beads to each accepted flask to assist quick washing. Rock the flasks gently until the growth is in suspension. Check washing from each flask for purity by microscopic examination of stained smears. Discard flasks showing contamination, if any. Pool suspensions from accepted production flasks in pre-sterilized Erlenmeyer flasks/ This constitutes a 'single harvest'/

Using formal saline, dilute the single harvest to match the opacity of the suspension equivalent to Brown's Opacity Scale 14. This constitutes the 'Final Bulk'/ Test sterility (inactivation test) of the bulk (Annexure-3; *below*). Before addition of adjuvant the bulk may be stored at 4°C.

2. Batch fermentation

Sterilize CSY broth (Annexure-2) in the Fermenter at 121°C for 45 minutes according to the facility available (*in situ* or in autoclave). Cool medium to 30°C and inoculate 50 ml of accepted working seed (*see* WSL) for 1 liter medium in the

Fermenter. Add sterilized auto-digest of pancreas (Annexure-2) at the rate of 40 ml / liter and 3 ml of sterilized antifoaming agent per 10 liter of the medium.

Connect air inlet with sterilized pre and absolute filters and adjust incubation temperature to 37°C. Start aeration and agitation of medium after 2 hours of inoculation. Introduce sterilized air through the medium at the rate of one-third volume of the medium per minute for the first 6 hours and half of the volume for remaining 10 hours. For the first 6 hour of incubation, adjust speed of the stirrer at 200 rpm and then increase it to 400 rpm till completion of incubation. Incubate for 18 hours and harvest the bacterial suspension in sterilized flasks of suitable capacity. Test the purity and morphological identity of the suspension in each flask. Pool the suspensions of accepted flasks.

Inactivate pure pooled suspension by adding sufficient quantity of commercial formaldehyde solution (37-40%) to give 0.5% final formalin concentration (v/v) of bacterial suspension. Shake the flasks and keep at 37°C for 24 hours. Check sterility of the formalinized suspension (Inactivation test; *below*).

Check the Brown's opacity equivalent of the culture suspension (may range between 18 and 21) and dilute suitably with sterilized formal saline to match Brown's opacity scale equivalent to 14/Store the standardized suspension at 4°C.

B.3. Adjuvantation

1. Aluminum hydroxide gel adjuvant vaccine

Adjust the pH of the standardized suspension with desired antigenic mass to 6.2-6.4 by 0.1N HCl (pH adjustment is critical for satisfactory adsorption of gel)**.** Add 40 parts of 3 percent (w/v) aluminium hydroxide gel (sterilized by autoclaving) to 60 parts of the suspension in a mixing tank and mix by agitating the mixtures slowly at room temperature (25°C) for 4 hours.

2. Oil adjuvant vaccine

In the Biological Products (BP) Division, ICAR-IVRI, 15 parts of suspension containing desired biomass of P_{52} is emulsified with 10 and 1 parts respectively of sterilized light liquid paraffin and pure anhydrous lanolin.

Liquid paraffin and lanolin are sterilized in hot air oven at 160°C for 1 hour and allowed to cool gradually over a period of 2-3 hours.

Sterilized liquid paraffin is transferred aseptically to a vaccine mixing tank and calculated quantity of lanolin is then added. Sufficient quantity of commercially available formalin solution is also added so that its final concentration in the paraffin-lanolin is 0.5% (v/v). Paraffin-lanolin is mixed at room temperature (25°C) for 20 minutes. Appropriate quantity of antigen suspension is then introduced slowly to paraffin mix and blended for 20 minutes at room temperature/ till a creamy-white, stable emulsion is produced.

Finally, the vaccine is re-emulsified similarly once again on the following day after keeping it at 4°C overnight.

C. QUALITY TESTING OF VACCINES

C.1. Visual examination of vaccine vials

Inspect every vial individually under proper illumination. Examine the vials against both white and black background. Discard containers showing gross foreign particles/aggregations.

C.2. Sterility

Test for absence of aerobic and anaerobic bacterial contamination is carried out in FTM (Annexure-2), and test for absence of fungi is carried out on SCDM (Annexure-2) as per IP 2018 guidelines/Schedule F1 recommended methodology.

1. **Test on batches**
   - **Test for absence of aerobic and anaerobic bacterial contamination**

     Inoculate 10 ml of the test material from a pool of vials into each of 2 bottles containing 150 ml of FTM (Annexure-1). Inoculate one bottle with standard bacterial cultures of *Cl. Sporogenes*, *S. aureus*, and *Ps. aeruginosa* as positive controls. Incubate them at 35-37°C. After 5 days, if required, subculture 1 ml from the bottles containing test material only, into another 150 ml container of FTM, and incubate at 37°C up to 14 days. Examine all containers on 3rd, 5th, 7th days and on the last day by microscopy of stained films prepared from the bottles.

   - **Test for absence of fungal contamination:**

     Inoculate 10 ml of the test material from a pool of vials into each of 2 bottles containing 150 ml of SCDM (Annexure-1). Incubate one of them at 37°C and the other at 25°C. Inoculate one bottle with standard fungal cultures of *Aspergillus brasiliensis*, *Candida albicans*, and *B. subtilis* as positive controls. After 5 days, if required, subculture 1 ml from each of the test bottles into another 150 ml containers of SCDM and incubate at 37°C and 25°C for 14 days. Examine all containers on 3rd, 5th, 7th days and, on the last day as described above.

The material/batch passes the test if there is no evidence of any bacterial and fungal growth provided samples of medium prepared along with the bottles used for testing, show positive growth promotion with the recommended organisms.

2. **Inactivation test on harvests**

   Test for inactivation is carried out on formalinized harvests by inoculating blood agar and nutrient or BHI broth (Annexure-2). No growth of the vaccine strain should be obtained on any of these media.

C.3. Safety tests

1. **For characterization of MSL (Target Animal Safety Test; TAST)**

   TAST is conducted only once for the characterization of seed lot.

   Inject at least 2 healthy buffalo or male cow claves with twice the dose of the product through appropriate route (subcutaneous for Alum gel or intramuscular

for oil adjuvant) and observe for 10 days. The seed lot passes the safety test if none of the animal shows any obvious adverse reaction and die of *P. multocida* infection.

2. On finished product during manufacturing process/batch

Inject each of a group of at least 6 healthy, adult mice with 0.5 ml of test preparation (final batch) through intraperitoneal route. Observe for 5 days for any post-vaccinal untoward reaction and / or mortality. Conduct post mortem of the dead animals (if any) to confirm cause of death. The batch passes the safety test if none of the animals show any obvious adverse reaction against vaccination and die of *P. multocida* infection.

C.4. Potency / Immunogenicity/ Identity tests

1. For characterization of seed lot (conducted once in target animal species)

(i) Selection of animals

Five healthy animals ageing between 8-12 months, which should be free from natural anti-*P.multocida* serum antibodies, are required for potency test. For selection of animals, conduct the following test for detecting absence of natural anti-*P.multocida* antibodies. Blood sample from each animal to be tested is collected through jugulovena puncture to separate serum (Fig. 10), and tested for presence of anti-*P.multocida* antibodies by plate agglutination test against freshly prepared live *P. multocida* (or P_{52}) antigen as per the steps mentioned below:

- **Preparation of live antigen for slide agglutination test:**

  Spread infected heart-blood or a freeze dried culture of the organism across 5-6 blood agar plates (90 mm diameter). Incubate at 37°C for 18 hours. Wash off the bacterial growth with about 4 ml NSS/plate and check the purity of each suspension by wet and stained film examinations. Pool pure suspensions and centrifuge for 2 minutes at 1000 rpm to remove coarse particles. Collect the supernatant bacterial suspension and centrifuge at 4000 rpm for 10 minutes. Discard supernatant. Re-suspend the pelleted cells in sufficient saline solution so as to match equivalent to with Brown's scale 24/ Use freshly prepared live antigen for slide agglutination test for selecting sero-negative cow or buffalo calves.

- **Test Proper (Slide agglutination Test)**

  Place a drop of (about 30µl) freshly prepared live antigen on a clean, grease-free glass slide. Add equal quantity of serum sample from the animal to be tested. Mix with a match-stick and observe in oblique, transmitted light. A coarse floccular agglutination within 60 seconds indicates a positive reaction.

  Animal showing absolute absence of natural anti-*P. multocida* serum antibodies is selected for potency tests.

(ii) Potency test proper

Inoculate 2 ml (animals having body weight <140 Kg); or 3 ml (animals having >140 Kg BW) of the test product (from 5 pooled vaccine sample containers) in three buffalo or cow calves (male), through the route recommended for vaccine. (Inject aluminium gel vaccine through subcutaneous route; if it is oil adjuvant vaccine, inject intramuscularly). Observe the animals for 21 days. Challenge the vaccinated animals along with 2 controls with **at least 50 million mouse minimum lethal dose** of challenge strain (For P_{52}, 1 ml of 1 in 10 dilution of 6-12h culture in nutrient or BHI broth) (Annexure-3; *below*) by subcutaneous route in the neck region. Observe these animals for 7 days.

The seed lot passes the test if at least 2 vaccinated animals out of the 3 immunized survive, while both controls succumb to the challenge infection

2. On finished product/batch (in mice)

Inject 50 mice of either sex weighing not less than 18 g, subcutaneously with 0.2 ml of Alum gel vaccine or intramuscularly with 0.2 ml of the oil adjuvant vaccine from pooled sample of 5 vials. Repeat the dose similarly after 14 days. After 7 day of the second vaccination divide the vaccinated mice into 10 groups of 5 each. Use 50 mice of same age from the same stock as controls and divide them into 10 groups of 5 each.

Challenge each of the vaccinated and the control mice of each group with 0.2 ml of a dilution of 12-18 h old broth culture of a virulent strain of *P. multocida* ranging from 10^{-1} to 10^{-10} through subcutaneous route. Observe the mice for 5 days and record the mortalities in vaccinated and control groups. Calculate the 50% lethal dose of the challenge organism (LD_{50}^{*}) for vaccinated and control mice by Spearman and Karber method. The protection provided by the vaccine is determined as Protective Index (PI), using following formula:

Protective Index (PI) = LD_{50} in Control mice ÷LD_{50} in vaccinated

The batch passes the test if PI is not less than $4\log_{10}$

3. Determination of mouse minimum lethal dose (MLD) and 50% lethal dose (LD_{50})

Use 50 healthy adult mice weighing not less than 18-20 g.; divide them in 10 groups, each having 5 animals. Prepare 10 fold dilutions of a pure, young (18-24 hour grown) nutrient broth culture of *P. multocida* ranging from 10^{-1} to 10^{-10}. Inoculate 0.2 ml of a dilution through subcutaneous route to each of a group of 5 mice. Observe for 5 days and record mortalities. Culture heart blood of each mouse immediately, or as soon as possible after death, to confirm presence of *P. multocida*. The highest dilution of culture showing 100% mortality in the challenged mice group is considered as mice MLD. 50% lethal dose (LD_{50}) of the strain may be determined by Spearman and

Karber (Karber,---)statistical method. Example of an experiment conducted in the Division of Standardization is given (Fig. 15).

A. Experiment

Mice: 50; healthy, adult; not less than 18-20 g

Challenge:

Culture: 6-12 hours growth in BHI broth

Dilutions of culture: 10^{-1}-10^{-10}

Challenge dose: 0.2 ml

Route of injection: S/C

No. of mice (n) /dilution 05

Duration:

Total days to observation: 05

Observation: Mortality of mice

B. Data on mortalities after termination of experiment

Dilution	Died	Survived	Mortality Ratio	Accumulated values			
				Death (D)	Survive (S)	Mortality ratio	% mortality
10^{-1}	5	0	5/5	40	0	40/40	100
10^{-2}	5	0	5/5	35	0	35/35	100
10^{-3}	5	0	5/5	30	0	30/30	100
10^{-4}	5	0	5/5	25	0	25/25	100
10^{-5}	5	0	5/5	20	0	20/20	100
10^{-6}	5	0	5/5	15	0	15/15	100
10^{-7}	5	0	5/5	10	0	10/10	100
10^{-8}	5	1	4/5	5	**10**	10/10	100
10^{-9}	1	4	1/5	1	9	1/10	10
10^{-10}	0	5	0/5	0	5	0/5	00

C. Determination of MLD and LD_{50}

1. **Mouse MLD** = 0.2 ml of 10^{-8} dilution of P_{52} through subcutaneous route
2. LD_{50} is determined by the following 'condensed formula'

$$LD_{50} = x + 0.5(d) - \frac{d\sum r1}{n}$$

x = Highest dilution tested **(10)**

d = Interval between successive log doses (dilution factor) **(1)**

$d\sum r1$ = Total number of survived hosts **(10)**

n = Number of mice used at each dilution **(5)**

LD_{50} = 8.5 or ($log^{8.5}$/0.2 ml)

Fig. 15. Example of determination of mouse minimum lethal dose (MLD) and 50% lethal dose (LD_{50})

ANNEXURE-4

Experimental Animals

A. MICE

1. Mice are used for safety and potency testing of Haemorrhagic septicaemia vaccines

2. Physiological data

Normal temperature:	37.4°C (99.3°F)
Pulse rate:	120/minute
Weight (adult):	18-25 g
Daily feed intake:	5.0-10.0 g
Room temperature:	20-21°C
Humidity:	50-60%

3. Housing and cages

The following guidelines of CPCSEA may help to determine the space needed for mice.

- Mice housed in groups should have a cage height of no less than 5 inches (12.7 cm) and a floor area per mouse of 6 to 15 inches (38.7 to 96.7 cm) squared, depending on body weight. A female mouse and her litter should have 51 square inches (330 cm) of floor space at a minimum. Cages should be light, durable and easily sterilized by dry or moist heat.
- An aluminium box (~6x12x6 in.) with tapering sides to allow stacking is preferred. The lid made of sheet metal or strong wire mesh and so designed that a food hopper is built into them and accommodation provided to hold the drinking water bottle. Similar cages of polypropylene (PP) may also be used. PP cages are less expensive, equally satisfactory and, can be sterilized in autoclave but not in hot air oven. Up to 6 mice can be housed in a cage of this type. For holding up to 100 mice, larger cages (~30x18x6 in.) can be used.

4. Feeding

Pelleted diets are satisfactory. Fresh water in drinking bottles should be provided *ad lib.*

5. Handling

With assistance

- An assistant grips on the middle of the tail of the animal with his left hand and gently raises the hind limbs from the floor of the cage. This is safe, as the mouse in this position cannot turn round and bite.
- With the right finger and thumb, a fold of skin is taken up as close as possible to the head and the animal is lifted into a convenient position for the operator.

Without assistance

- Place the animal on a rough surface and hold its tail with the right hand.
- Pick up the loose skin at the base of the neck with the left forefinger and thumb.
- Lift and turn the left hand palm uppermost at the same time catching the tail and pressing it against the palm with the left little finger making the right hand free to pick up and hold a syringe.

6. Inoculations

Subcutaneous (S/C; s/c)

- An assistant holds mouse in a steady position by grasping loose skin at the nape of the neck in one hand and the tail in the other.

- Needle is introduced under the skin near the root of the tail and the vaccine or culture may be injected in the desired amount.

Intraperitoneal:

- An assistant must hold the animal as described above and then turn it over. For steadiness he should rest his arms on the working table.
- The injection is made to the one side of the mid line in the lower half of the abdomen.

7. Post-mortems and collection of heart blood

Dead animal or deep anaesthetized animal is pinned out and the skin over thorax and abdomen is reflected to expose the heart. Blood is collected in a **sterile** Pasteur pipette.

8. Common diseases

Intestinal infections due to *Salmonella* Enteritidis and *S.* Typhimurium, (termed as mouse typhoid), may produce severe epizootics. The stock should be destroyed, cages disinfected and fresh stock obtained.

B. RABBIT

1. Uses

Potency test of HS Vaccines; Raising of antisera

2. Physiological data

Normal temperature:	38.0-39.7°C (100.5-103.5°F)
Pulse rate:	120-150/minute
Respiratory rate:	38-60/minute
Average body weight (adult):	Male (1000-1200g up to 4000g); Female (mother with kids up to 5400g)
Total blood volume:	70ml/kg body weight
Available blood volume:	35 ml/kg body weight
Clotting time	60-300 minutes
Daily feed intake:	~120 g (100-200g)
Daily water intake:	20 ml
Room temperature:	62-68°F (16.7-20°C)
Humidity:	45-55 %

3. Housing and cages

Adult rabbits are housed **individually** in metal (preferably stainless steel cages) with wire mesh floors, with excreta collection trays below. For a rabbit weighing less than 2000g, minimum floor area recommended by CPCSEA is 1.5 sq. ft (0.135 sq meters); the height of the cage should not be less than 14 inches. For maintaining rabbits free of respiratory and other diseases (*below*), adequate and free ventilation is the most important requirement.

4. Feeding and nutrition

Rabbits are herbivorous. The animals can be adequately maintained on any one of the numerous complete pelleted rations widely available commercially. Fresh water should be provided daily and *ad lib.* Hay supplements may be provided.

5. **Handling**

When removing a rabbit from a cage, or picking it up, the **loose skin over the shoulders can be grasped** with one hand. The other hand also grasps the loose skin along the back (in large rabbits) or supports the abdomen. Rabbits should **never be picked up by the ears** as these are easily hurt and injured.

6. **Inoculations**

Subcutaneous:

- Subcutaneous inoculation may be made either into the abdominal wall or into the loose tissue about the flank or at the back of the neck.
- The hair is clipped, and the skin is sterilized either with 70% alcohol or iodine and pinched up. Needle is introduced under the skin and test material may be injected in the desired amount.

7. **Collection of blood**

About 10-15 ml of blood may be easily obtained from the ear vein of a large rabbit (weighing ~ 1000 g) **without causing any distress to the animal**. The **marginal vein of the ear is the most convenient site**.

- The rabbit may be held by an assistant. **In the absence of assistant**, the animal may be restrained by placing it in a special box or **wrapping in a towel** so that only its head protrudes.
- The hair over the vein is **dry shaved** with a sharp razor; and the vein may be distended either by vigorous rubbing with a piece of cotton-wool or by holding the ear over an electric light bulb, when the heat causes dilatation of blood vessels.
- A sterile syringe of 10 ml capacity with a long needle of No. 20 Gauge may be used.
- The operator faces the animal and the ear is held horizontally by means of the left hand. The needle is kept as nearly parallel as possible to the vein. The point is inserted towards the head of the animal and required volume of blood is slowly drawn.
- When collection is completed, the needle is withdrawn and a small piece of cotton-wool is paced on the vein, which is then compressed between the thumb and the finger.

Water should always be provided in the cage after bleeding.

8. **Common diseases**

Rabbits should be obtained from breeding colonies that are free from *P. multocida* infection. Rabbits may be affected by a wide variety of infectious diseases that may interfere with their use in vaccine testing and research. Diseases of the respiratory tract and intestinal diseases with diarrhea are particularly common.

- **Respiratory infections:** Snuffles caused by *P.multocida* is most common. Affected rabbits exhibit a purulent discharge from the nares and/or eyes, with sneezing. Pasteurellosis may also give rise to middle ear infection (otitis media) with the eventual development of a wry neck (torticollis); when this develops, treatment will be of no value and affected rabbits should be humanely killed.
- **Intestinal infections:** Diarrhea, resulting from intestinal infections caused by organisms such as *Salmonella* Typhimurium are common; however, in many cases the etiology remains unknown. Intestinal coccidiosis and Tyzzer's Disease may be the causes of intestinal diseases. **Coccidiosis is a common disease of rabbits.**
- **Parasitic infestations**: Parasitic mange due to mite usually affecting ears; and ***Taenia pisiformis*** (cysticercus stage of the dog tape-worm) infestation causing numerous cysts in the omentum and sometimes liver are the most common parasitic diseases observed in rabbits.

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