Himachal Journal of Agricultural Research 40(2):99-109 (2014)

Pasteurellosis in animals of Himachal Pradesh

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Manuscript Received: 01.04.2014; Accepted: 03.06.2014

Abstract

Pasteurella multocida **is responsible for major animal diseases of economic importance in both developed and developing countries including India. The prevalence of pasteurellosis has been documented in India as well as in the state of Himachal Pradesh where disease outbreaks from different agro-climatic regions have been reported. Aiming at increased knowledge of these infections in Himachal Pradesh, we employed the conventional methods and advanced molecular methods for culturing, characterization, capsular serotyping, and analysis of virulence factors for rapid epidemiological investigation and pathogenicity of** ***Pasteurella multocida*** **infections in H.P. Vaccine-induced protective biomarkers were also studied using advanced immunological techniques. We also summarize our efforts to enhance the ability to rapidly diagnose clinical infections and to prevent and control pasteurellosis by validation of vaccines.**

Key words: Haemorrhagic Septicaemia, *Pasteurella multocida*, diagnostics, typing, virulence factors, vaccines.

Introduction

Pasteurellosis is a global problem, including India and has been reported from livestock, birds and a variety of wild animals. Pasteurellosis caused by Gram-negative bacterium, *Pasteurella multocida* and *Pasteurella haemolytica*, recently renamed *Mannhemia haemolytica* encompasses a variety of disease syndromes in numerous domestic animal species. *P. multocida* has been classified into three subspecies, five capsular serogroups, and 16 serotypes. The various serotypes of *P. multocida* have been associated with a wide range of diseases such assepticemia, respiratory distress, rhinitis, conjunctivitis, and mastitis, collectively termed as pasteurellosis. *P. multocida* causes septicaemia in cattle, pigs, birds and rabbits; bronchopneumonia in cattle, sheep, goats, pigs, rats and rabbits (snuffles); rhinitis in piglets and rams; wound infection in dogs, cats and humans, and fowl cholera in birds. The disease is an acute, highly fatal septicaemic disease of cattle and buffaloes and is caused by certain serotypes of *Pasteurella multocida*. Asian serotype is designated as 6:B or B:2 and African serotype as 6:E or E:2. Serotyping of isolates from different animal species also reveals the prevalence of other serotypes in cattle like A: 1, A: 3, B: 2, F: 3; buffaloes like A:3, B:2, B:5; sheep like A:3, D:3, F:1, F:4, F:10; poultry as A:1, B:2, B:5; ducks as F:3; lions as A:3, A:4; tiger as A:3, A:4; deer as A:2, A:5. *M. haemolytica* causes fulminating fibrinous pneumonia known as shipping

fever and *Pasteurella* are normally considered as commensals of upper respiratory tract of ruminants. Pasteurellosis flares up owing to overcrowding, chilling, prolonged transportation, fatigue, starvation, malnutrition, climatic changes or inter-current infections. Haemorrhagic Septicaemia (HS) is a primary pasteurellosis with 100% mortality in infected animals in endemic areas of Africa and Asia and has been classified as a list B disease by the Office International des Epizooties (OIE). In India, HS has been documented to be one of the major bacterial killer diseases among cattle and buffaloes. In the state of Himachal Pradesh, no comprehensive information exists about animal pasteurellosis and associated economic losses before the year 2000. However, a number of studies performed in our department have identified the presence of *P. multocida* and corresponding clinical diseases among cattle, buffaloes, sheep, goats, rabbits, and poultry.

Detection, identification and characterization of *P. multocida*

For detection of infection with *P. multocida*, samples to be collected from live animals are whole blood and nasal secretions and from dead animals are lung, liver, spleen, kidney, bone marrow of long bones, and heart tissue. Table 1 summarizes various clinical samples collected by our department.

Culture and biochemical characterization

Conventional methods for detection and identification of infection with *Pasteurella* rely on observation of bacterium by microscopy, isolation by culturing and biochemical characterization.*P. multocida* is a small (0.2-0.4 x 0.6-2.5 μm), Gram-negative, non-flagellated, non-spore forming coccobacillus. Microscopic examination of fresh cultures or clinical specimens reveals characteristic bipolar-staining rods with methylene blue, Leishman or Giemsa stain.Isolates of *P. multocida* are aerobic or facultative anaerobic and grow well at 37°C on 5% sheep blood (most common), dextrose starch, casein-sucrose yeast (CSY), chocolate, Mueller-Hinton, or brain heart infusion (BHI) agar; however, there is no growth on MacConkey agar. Most clinical isolates are catalase, oxidase, indole and nitrate reduction positive. Most isolates also ferment sucrose, glucose, and maltose. A summary of various clinical isolates obtained in our departmentis detailed in Table 1.

Molecular characterization

P. multocida isolates are classified into five serogroups based on capsular polysaccharide phenotyping as serogroup A (hyaluronic acid), B (arabinose, mannose, and galactose), D (heparin) , E (uncharacterized), and F (chondroitin) . Various isolates of *P. multocida* are thus designated according to their capsular serogroup followed by a somatic serovar number e.g. A:1, A:2, A:3, B:1, B:2, etc..Isolates are also subtyped based on their lipopolysaccharide (LPS), which separates isolates further into 16 serovars . PCR-plus sequence-based ribotyping analysis using universal primers for 16S rRNA genes, genomics and other DNA sequence-based molecular techniques have now superseded phenotypic methods for identification, detection, and characterization of *P. multocida*.A rapid *P. multocida*-specific PCR (PM-PCR) assay for the species identification and a multiplex PCR typing system for rapid capsular serotyping have been developed and applied worldwide . Both of these assays have been standardized in our department for the rapid confirmation and serotyping of *P. multocida* isolates. Using PM-PCR assay, *P. multocida* isolates were identified based on the amplification of an approximately 460 bp fragment . The capsular types of the isolates obtained in our lab were determined by multiplex capsular PCR that identified target strains by the amplification of an approximately 1044 (type A), 760 (type B), 657 (type D), 511 (type E), and 851 (type F) bp fragments .

***Pasteurella* disease in animals**

Pasteurellosis prevalence

Pasteurella species cause many endemic and epizootic diseases in a wide range of domestic and wild animals, including birds and are have a worldwide distribution. *P. multocida* is a common

Table 1. A summary of number of samples processed and *P. multocida* isolates obtained from different animal species in Himachal Pradesh

Year	Cattle		Buffaloes		Sheep		Goats		Rabbits		Poultry		Equine		Monkey		Pig		Neelgai		Leopard		Total	
	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I
2000-01	45	2	16	4	25	0	6	0	110	2	-	-	-	-	-	-	-	-	-	-	-	-	**202**	**8**
2001-02	99	4	38	0	276	3	275	4	490	10	-	-	-	-	-	-	-	-	-	-	-	-	**1178**	**21**
2002-03	56	2	23	0	155	2	191	3	253	5	-	-	-	-	-	-	-	-	-	-	-	-	**678**	**12**
2003-04	63	3	37	1	105	0	143	2	217	3	-	-	-	-	-	-	-	-	-	-	-	-	**565**	**9**
2004-05	35	6	71	19	115	1	214	1	116	1	-	-	-	-	-	-	11	0	-	-	-	-	**562**	**28**
2005-06	109	3	38	2	57	0	129	0	125	1	-	-	2	0	-	-	3	0	2	0	-	-	**465**	**6**
2006-07	40	4	48	10	46	0	95	0	262	3	-	-	4	0	-	-	-	-	-	-	9	0	**504**	**17**
2007-08	127	3	62	8	22	0	56	0	236	2	182	8	6	0	-	-	-	-	-	-	-	-	**691**	**21**
2008-09	164	4	27	1	44	0	79	0	209	3	16	0	-	-	-	-	-	-	-	-	-	-	**539**	**8**
2009-10	255	16	15	2	28	0	52	0	133	7	52	0	-	-	-	-	-	-	-	-	-	-	**535**	**25**
2010-11	73	14	37	3	57	0	40	0	263	3	28	0	-	-	25	0	-	-	-	-	-	-	**523**	**20**
2011-12	80	1	18	0	38	1	57	0	124	2	18	0	4	0	-	-	-	-	-	-	8	0	**347**	**4**
2012-13	179	4	64	1	59	0	83	0	43	0	49	0	-	-	-	-	-	-	-	-	-	-	**477**	**5**
	1325	**66**	**494**	**51**	**1027**	**7**	**1420**	**10**	**2581**	**42**	**345**	**8**	**16**	**0**	**25**	**0**	**14**	**0**	**2**	**0**	**17**	**0**	**7266**	**184**

S= Samples processed ; I = Isolates of *P.multocida*

commensal or opportunistic pathogen of the upper respiratory tract of most livestock, domestic and wild animals, including cattle and buffaloes, swines, rabbits, chickens, turkeys, other wild birds, goats, chimpanzees, and marine animals. The pathological manifestations of pasteurellosis range from asymptomatic or mild chronic upper respiratory tract inflammation to acute, pneumonic and/or disseminated disease. Transmission occurs through direct contact with nasal secretions, where a chronic infection develops in the upper respiratory tract and lungs. Infection with respiratory viruses, *Mycoplasma* species or coinfection with respiratory pathogens especially *Bordetella bronchiseptica* or *Mannheimia haemolytica*, predispose the animals to secondary infection with *P. multocida.* In addition, environmental conditions and the immune status of the animal also determine the susceptibility of the animals to *P. multocida* .

Pasteurellosis pneumonia

Pasteurellosis in animals is predominantly exhibited as an upper respiratory disease in the form of rhinitis (inflammation of nasal mucosa and nasal secretions) and lower respiratory disease in the form of pneumonia (in cattle referred to as the bovine respiratory distress syndrome). Symptoms of pasteurellosis range from sneezing, mucus secretions, rhinitis, pneumonia with laboured breathing, fever but can also progress to disseminated disease as in HS. *P. multocida* is often endemic in rabbit colonies and swine herds where the disease condition is called snuffles. Snuffles is characterized by sneezing, snuffling, nasal discharge, teary eyes, and lead to twisting, wrinkling and distortion of the snout .

Pasteurellosis and Haemorrhagic Septicemia

Haemorrhagic Septicemia caused primarily by *P. multocida* serotypes B:2 and E:2 is a serious acute, highly fatal, and highly prevalent disease in livestock, especially cattle and buffaloes in Asia, India, Africa, Southern Europe and the Middle East . The disease occurs in acute, sub-acute and chronic forms. The acute form of HS is characterized by rapid onset (within a few hours) and death within 24 hours. Animals show a high rise in body temperature, rapid respiration and cyanosis of mucus membranes . In the sub-acute form of HS, animal survives for 2-3 days with oedematous swelling of the throat and brisket region and bronchopneumonia. Chronic form of the disease is characterized by rapid painful respiration and mucopurulent or blood stained respiratory discharge. Postmortem examination reveals subcutaneous oedema in the mandibular and brisket regions, haemorrhages, congestion and consolidation of lungs, fibrinous pneumonia, pleurisy and pericarditis .

Pasteurellosis and fowl cholera

P. multocida capsular serotype A, mainly A:1, A:3, and A:4 are responsible for fowl cholera, although serotypes F and D have also been reported. The respiratory tract appears to be the primary site of infection, however *P. multocida* has also been isolated from avian salpingitis. Fowl cholera is characterized by an asymptomatic or mild chronic sinusitis and conjunctivitis or pneumonia-like pasteurellosis, but can also develop into fatal disseminated disease . In acute cases, green diarrhoea is an early symptom while in chronic cases the most prominent symptom is the swelling of the wattles.

***P. multocida* virulence mechanisms**

P. multocida has several mechanisms or components that are involved in enhancing survival in the host environment and evading the host immune response, collectively called virulence factors .These virulence factors can be: iron acquisition mechanisms that enable *in vivo* growth, membrane lipopolysaccharide (LPS) that confers resistance, capsule that prevents phagocytosis, OMPs that enhance colonization, extracellular matrix degrading enzymes such as hyaluronisae, neuraminidase, and proteases that facilitate colonization and dissemination, *P. multocida* toxin (PMT) that causes dermonecrosis, and plasmids that confer antibiotic resistance or encode for various virulence attributes including

toxins. Nearly 104 putative virulence-associated genes that account for only 7% of the coding density of the *P. multocida* genome have been predicted . This shows that a large number of virulence factors are still to be discovered. Out of 184 *P. multocida* cultures isolated in the department, 23 isolates were characterized by uniplex PCR method for 11 virulence-associated genes that included genes encoding for iron acquisition factors (*hgbA, hgbB and tbpA*), adhesion related genes (*ptfA, nanB, nanH, and pfhA*), outer membrane and porin proteins (*oma87*), superoxide dismutases (*soda and sodC*), and dermonecrotoxin (*toxA*). We also correlated the distribution of virulence-associated genes in *P. multocida* based on the health status of animals. The OMPs have a role in disease processes by acting at the interface between host and pathogen. Amongst OMPs, OmpA protein is a major structural component of the outer membrane of the gram-negative bacteria. Isolates of *P. multocida* were characterized for *ompA* gene diversity through a PmompA PCR standardized in the department. The identified ompA classes of *P. multocida* were then associated with capsular types in a particular pattern. OmpA major classes I to IV were associated with isolates harbouring capsular type B while class V have isolates mostly belonging to capsular type A barring few exceptions. All the isolates harbouring capsular type A were associated with ompA 6.1-type allele of major class V, while the isolates with capsular type B were associated with ompA 6.2- and 6.3- type alleles. Most recently our group has shown that *P. multocida* strain with ompA (I) is more invasive than *P. multocida* strain with ompA (II) following *in vitro* pathogenicity studies on Madin Darby Bovine Kidney (MDBK) cell lines and supported by findings from *in vivo* studies in mice.

Chemotherapy and antibiotic resistance

Treatment could be effective only in the early stages of *P. multocida* infection. Generally, clinical cases of HS are extensively treated with oxytetracycline, co-triamoxazole, and a combination of penicillin and streptomycin or sulfaquinoxaline. However, in the past many years there has been an increase in the antibiotic resistance pattern that has lead to high disease mortality and associated economic losses . Many of the *P. multocida* isolates have been found to harbor plasmids, which confer resistance to various antibiotics, most frequently -lactams, tetracycline, chloramphenicol, streptomycin, and sulfonamides.Multiple antibiotic resistance genes have been identified integrated into the chromosomes of some strains of *P. multocida* e.g. ICEPmul, an integrative conjugative element (ICE). In our lab, we have accessed the drug resistance patterns of the *P. multocida* isolates over the years through standard disc diffusion assay (Table 2). Among the -lactam antibiotics, ampicillin, cloxacilin, cefuroxime, and ceftiofur were found to exhibit a high degree of resistance. While, streptomycin and tetracycline showed intermediate levels of resistance. These findings warrant a check on the indiscriminate use of antibiotics for the treatment of the disease in the state.

Prevention/Vaccination

Vaccination of susceptible animals in endemic areas like Indian subcontinent is the only practical approach to prevent large-scale HS outbreaks and infection with *P. multocida*. A number of vaccines are available in most countries including India to prevent the onset of disease. These comprise of both killed as well as live vaccines. Nowadays, there is a lot of emphasis on molecular approaches in the development of vaccines such as recombinant subunit vaccines, synthetic peptide vaccines, bacterial ghost vaccines, and mutant vaccines. Many outer membrane proteins of *P. multocida* have been targeted as potential vaccine candidates e.g. Oma87 , porin (OmpH), adhesion protein Cp39. Fimbrial protein from serotype B:2 have been found to be protective against HS in goats. Additional vaccine candidates include the filamentous hemagglutinin protein (FhaB2), LPS, lipoprotein E (plpE), hemebinding proteins (HasR, HemR, and HgbA) and transferring binding protein (TbpA). In our lab, we

Table 2. Drug sensitivity profile of clinical *P. multocida* isolates in Himachal Pradesh

S. No.	Antibiotics	Profile	No. of *P. multocida* isolates	Percentage of isolate drug resistance/sensitivity
1.	Amoxycillin	Resistant	24	13.4
		Intermediate	49	27.2
		High	107	59.5
2.	Ampicillin	Resistant	75	41.4
		Intermediate	27	15.0
		High	79	43.6
3.	Amikacin	Resistant	46	25.3
		Intermediate	65	35.7
		High	71	39.0
4.	Cephalexin	Resistant	23	12.8
		Intermediate	61	34.0
		High	95	53.0
5.	Ceftiofur	Resistant	107	59.7
		Intermediate	49	27.4
		High	23	13.0
6.	Cefuroxime	Resistant	87	48.6
		Intermediate	62	34.6
		High	30	16.7
7.	Chloramphenicol	Resistant	33	18.4
		Intermediate	49	27.4
		High	97	54.1
8.	Chlortetracycline	Resistant	85	46.4
		Intermediate	37	20.2
		High	61	33.4
9.	Ciprofloxacin	Resistant	17	10.6
		Intermediate	43	24.0
		High	119	66.5
10.	Cloxacillin	Resistant	93	50.5
		Intermediate	54	29.3
		High	37	20.1
11.	Doxycycline	Resistant	20	11.0
		Intermediate	91	49.5
		High	73	39.6
12.	Erythromycin	Resistant	26	14.1
		Intermediate	67	36.4
		High	91	49.4

S. No.	Antibiotics	Profile	No. of *P. multocida* isolates	Percentage of isolate drug resistance/sensitivity
13.	Enrofloxacin	Resistant	27	14.6
		Intermediate	79	43.0
		High	78	42.4
14.	Gentamicin	Resistant	39	21.2
		Intermediate	85	46.2
		High	60	32.6
15.	Tetracycline	Resistant	39	21.2
		Intermediate	63	34.2
		High	82	44.5
16.	Norfloxacin	Resistant	23	12.5
		Intermediate	95	52.0
		High	65	35.5
17.	Ofloxacin	Resistant	19	10.5
		Intermediate	73	40.3
		High	89	49.2
18.	Penicillin	Resistant	61	33.1
		Intermediate	54	29.3
		High	69	37.5
19.	Streptomycin	Resistant	37	20.1
		Intermediate	86	46.7
		High	61	33.1
20.	Spectinomycin	Resistant	39	22.5
		Intermediate	51	29.5
		High	89	51.5

compared the efficacy of two killed vaccines namely HS saponified vaccine that has been developed by Indian Veterinary Research Institute (IVRI), Bareilly and a commercial Oil Adjuvant Vaccine (OAV) through passive mouse protection test and evaluated the mechanism of vaccine-induced immune response using flow cytometry. Preliminary results show that in response to the HS saponified vaccine, CD4+, CD8+ and T cells (WC1+) were upregulated in the peripheral blood mononuclear cells (PBMCs) at 1 and 6 months post-vaccination, which corroborated with the results of passive mouse protection test following *P. multocida* infection.

Future perspectives

Advanced genetic, biochemical, and virulence studies of *P. multocida* and other *Pasteurellaceae* have provided valuable insights into the pathogenicity of disease and has led to the development of novel vaccines. However, there is insufficient data on incidence and distribution of HS in the country, which along with inefficient control measures has contributed to huge losses to the livestock sector. Therefore, in addition to further research on understanding the molecular virulence

mechanisms of *P. multocida*, it is essential to invest time and resources on disease monitoring and surveillance, extension services, and incentives to farmers through subsidies. For a state like Himachal Pradesh whose economy is dependent on agriculture and animal husbandry, adequate extension services and compensation to farmers is essential. In terms of research, an important area of focus is the whole genome sequencing and *in vivo* transcriptional and protein expression profiling studies during natural infections with B:2 or E:2 strains of *P. multocida*. These studies will help in a better understanding of host-pathogen interaction in *P. multocida* and will provide valuable insights into the transition from subclinical or chronic disease to acute, disseminated disease. Development of new vaccines also poses the challenge of their safe use in the animals and identification of markers of protective efficacy during the experimental trials. In a nutshell, current areas of research focus involving studies on disease pathogenicity and development of novel effective prophylactics and therapeutics have the potential to mitigate the devastating impact of pasteurellosis in the livestock sector.

Acknowledgements

The authors would like to acknowledge Indian Council of Agricultural Research (ICAR) for supporting our department through All-India Network Program on Hemorrhagic Septicemia (AINP-HS) (Sanction No. ICAR-303-V-40). We would also like to acknowledge scientists, postgraduate students, project, and technical staff that have been associated with this project since its inception.

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