Science World Journal Vol 9 (No 2) 2014
www.scienceworldjournal.org
ISSN 1597-6343

Full Length Research Article

EFFORTS TOWARDS THE DEVELOPMENT OF RECOMBINANT VACCINES AGAINST PASTEURELLA MULTOCIDA

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ABSTRACT
Hemorrhagic septicemia is caused by gram-negative bacterium of Pasteurella multocida (P. multocida) strains. Most of the current vaccines against P. multocida have shortcomings. Presently, there is increasing efforts towards construction of recombinant clone for vaccine development against P. multocida. In this review an effort is made to look at some strong candidate genes, different protein bands from virulent strains and recent reported recombinant antigens. The possibility of developing a broad spectrum or cross protection vaccine for P. multocida is also discussed. It is hoped that with the current development of three (3) completed genome sequence of P. multocida, stronger potential immunogens with broader spectrum will be identified. Furthermore whole genome sequence of other P. multocida strains will surely bridge the gap between diagnosis and vaccine development.

Key words: Virulent, Vaccine, P. multocida, immunogens.

INTRODUCTION
Haemorrhagic septicaemia (HS) is caused by the gram-negative bacterium *P. multocida* (Tabatabai *et al.,* 2007). The infection can be seen in both humans and animals with varying type of pathogenicity. In human it can be seen in soft tissue infection especially in elderly and immuno-compromised individuals (Baillot *et al.,* 2011). In animals, infection occurs due to ingestion of infected grass and water or inhalation. Haemorrhagic septicaemia (HS) is an acute and often fatal disease primarily occurring in water buffaloes and cattle, but occasionally in other domesticated and wild mammals (De Alwis, 1999). HS contributes to high rate of mortality in livestock animals, where it can spread among endemic and non-endemic area. Huge economic loss has been caused by the disease especially in tropical regions (Shivachandra *et al.,* 2011). The subspecie of *Pasteurella* are *multocida*, *septica* and *gallicida* (Kuhnert &Korczak,2006). *P. multocida* is the major subspecie of economic importance and it has been categorized into five capsular sero groups and 16 serotypes. Relationship exists between these capsular types and pathogenicity (Boyce & Adler, 2000). The sero groups identified are A, B, D, E and F. These serogroups were detected among the livestock population (Kumar *et al.,* 2004). Serotypes B: 2 and E: 2 are two common serotypes of *P. multocida* associated with disease in animals in Asia and Africa, respectively (Benkirane & De Alwis., 2002). Other sero groups afflict different species and may vary in virulence factors (Harper *et al.,* 2006). HS associated with serotype B:2 are distributed widely in Asia (Ataei *et al.,* 2009; Hajikolaei *et al.,* 2008). Buffaloes are more susceptible to HS than cattle (De Alwis., 1999). Studies by Ewers *et al.,* (2006) have shown that most of the virulence-associated genes in *P. multocida* are regularly distributed among strains from different hosts and disease status. they are not pathogenic (Verma & Jaiswal, 1998; De Alwis, 1999). At present, there is no commercial recombinant HS Presently researchers are taking different approaches towards the control and spread of the disease, including the development of subunit vaccine, using native or recombinant antigens (Hatfaludi *et al.,* 2010; Shivachandra *et al.,* 2011). This review is aimed at discussing various virulent factors and recombinant proteins. The possibility of determining the strongest candidate genes/proteins for vaccine development is also discussed.

Virulent Factors
The development of a strong antigenic agent needs proper understanding on different virulent factors. Among the virulent factors is the outer membrane protein which includes the porins, receptors, and pores. Some of the outer membrane proteins are called porins due to their role in cellular permeability (Benz, 1988). It has been reported that pathogenic bacteria produce more vesicles than their nonpathogenic counterparts (Liu *et al.,* 2012). The secretion by gram negative pathogenic bacteria enables it survives in its host cell. The Lipopolysaccharides (LPS) are also included as part of the virulent factors. Another virulent factor is the capsules which have been known to cause HS in buffalos. The classification of *P. multocida* into capsular types is based on their composition and they play a pivotal role in pathogenicity. Other virulence factors include the fimbrae which has been isolated from *P. multocida* serotype A, B and D with ability to adhere to mucosal epithelium (Ruffalo *et al.,* 1997). Extracellular enzymes such as the hyaluronidase lipase, and neuraminidase or sialidase are associated with virulence of *P. multocida* (Ruffalo *et al.,* 1997). High levels of these enzymes may probably indicate increase in virulence.

Vaccine Development
Live-attenuated micro-organisms, inactivated bacteria, purified microbial components, polysaccharide- carrier protein conjugates, recombinant proteins or DNA are used as vaccines (Freddy *et al.,* 2004). One of the most widely used vaccines in Asia is the whole cell formalin killed *P. multocida* P52 bacterin emulsified in aluminium hydroxide gel (Qureshi & Saxena, 2014). This vaccine being used, however, does not provide long immunity (Qureshi & Saxena, 2014). It is therefore obvious that there are difficulties being encountered in producing vaccines for *P. multocida* this may be due to the high antigenic variability and low reported work on whole genome sequence. In addition the development of vaccines involves the detailed understanding of the mechanisms and mode of action of those virulent factors. Various studies have shown inconsistent efficacy of these vaccines (Moiser *et al.,* 1994). Other challenges being encountered in the determination of a strong candidate gene from the pool of potential genes discovered, is the need to test the protective properties on animal model. Furthermore potential gene products or protein will have to be purified and characterized before its antigenic properties can be fully known.

The use of recombinant technology for vaccine development may soon revolutionize the current vaccines. Recombinant antigens can be obtained from different expression systems after the targeted antigen has been determined. Recombinant proteins used as subunit vaccines have been reported safe as subunit vaccine (Hussaini *et al.,* 2012). The cross-protective antigens still remain elusive (Shivachandra *et al.,* 2011).

Therefore recombinant technology seems promising and may be used to develop an efficient vaccine towards protection against most strains of *P. multocida*. Stronger and broad spectrum vaccine through identifying conserved immunogenes can be achieved from various identified strains of *P. multocida*.

Candidate Genes

Several candidates genes against *P. multocida*. vaccines have been reported (Table 1). The stronger candidate genes having more resistant protection can be cloned or fused via recombinant technology to generate a stronger and more networking protective immunogens for the next generation vaccines.

Table 1: Potential candidate genes

Virulent gene	Protein	Major Protein Identified (Size) kDa	Sero Group	Reference
plpB	Lipoprotein B	39	A	Rimler, 2001. Tabatabai *et al.,* 2004, Chomnawang *et al.,* 2009 & Wu *et al.,* 2007
plpB	Lipoprotein B	28	A	Rimler, 2001, Wu *et al.,* 2007,
plpE	Lipoprotein E	37.4-37.7	X-73	Champlin *et al.,* 2002 and Nsofor *et al.,* 2006
plp-40	Lipoprotein E	40	A	Nsofor *et al.,* 2006
PMT	P. multocida toxin	146	A, D	Frandsen *et al.,* 1991; Williamson, 1994
OmpA	Outer membrane protein A	28- 36	N/A	Confer & Ayalew, 2013
OmpH	Outer membrane protein H	37.5	D	Dabo *et al.,* 2007 Shivachandra *et al.,* 2013.
OmpH	Outer membrane protein	33.6-38.5	D	Boyce *et al.,* 2006
Momp	Major outer membrane protein	32	D	Pati *et al.,* 1996
Ptfa	Type IV fimbrial subunit protein	14.9	A,B,D and F	Hatfaludi *et al.,* 2010

A total of 100 virulent genes in *P. multocida* have been reported (May *et al.,* 2001). One of the major candidate genes is the PMT gene. A study have confirmed that the toxigenicity of *P. multocida* is mainly derived from PMT, the PMT is encoded by tox A gene, establishing the fact that Tox A knockout gene contributes the major part of the toxigenicity of *P. multocida* (Kim *et al.,* 2006). Non Toxigenic *P. multocida* toxin (*PMT*) was created as candidate for use in vaccines against progressive atrophic rhinitis in pigs, this was done by replacing the serine at position 1164 with alanine (S1164A) and the cysteine at position 1165 with serine (C1165S) as such genetically modified PMT may represent a good candidate for use in developing a vaccine against progressive atrophic rhinitis in pigs. (Shivachandra *et al.,* 2005). Another putative candidate gene is the *ompA gene* (encoding an outer membrane protein) has also been reported to be involved in the adherence of *P. multocida* to host cells (Dabo *et al.,* 2003; Hatfaludi *et al.,* 2010). Several other Omp's have been identified including Omp16, Omp87 and iron-regulated OMPs such as TbpA, HemR, HbpA, HasR, HgbA (Wheeler *et al.,* 2009). Analysis of Omph from different serotypes showed a high degree of conservation (Meredith *et al.,* 2000). OmpH was shown to be of importance in stimulating immunity to infection

in animals (Davies & Lee, 2004). OmpA proteins are under evaluation as potential vaccine candidates (Shivachandra *et al.,* 2013). The OmpA, will be a favorable gene for the development of naturally and recombinant-derived vaccines (Shivachandra *et al.,* 2013).

The ptfA gene has also been proven to have a positive association with the disease outcome in cattle and hence could be used to develop broad cross-protective and disease specific subunit vaccine along with suitable adjuvants especially for HS in bovines and septicaemic pasteurellosis in sheep and goat (Shivachandra *et al.,* 2013). Among the proposed candidate gene is the plpB gene, although controversies have surrounded the acceptance of the plpB as a candidate gene for the development of *P. multocida* vaccine. It was first purified and proved to confer protection against different serotypes of PM (Rimler, 2001). The protein was confirmed to be *P. multocida* lipoprotein B by Tabatabai & Zehr (2004). Other reported candidate genes include the Aro A gene, as deletion of Aro A (A gene encoding the *P. multocida* toxin) of sero type B2 was used as an effective vaccine against HS (Ataei *et al.,* 2009). Another candidate gene is the CexA gene mutant (PBA875) which conferred high protection against *P. multocida* infection (Boyce *et al.,* 1991). Furthermore, one of the genes predicted to be involved in polysaccharide biosynthesis (bcbH *gene)* has been shown to confer significant protection against wild type

after immunization of mice with the live AL18 strain (Boyce & Adler, 2001). Other reported virulent genes are tbpA and pfhA as well as capsule biosynthesis genes are supposed to be important epidemiological marker genes for characterizing *P. multocida* field strains (Ataei *et al.,* 2009). Study by Ewers et al., (2006) confirmed that tbpa and pfha are virulent genes. The TbpA gene (82 kDa protein) first isolated in haemorrhagic septicaemia (HS) strains of capsule serotype B: 2, 5 in buffaloes and cattle (Veken *et al.,* 1994) have been shown to have strong homology with A1 and D1 (Shivachandra *et al.,* 2005).

Recent study by Verma *et al.,* (2013) found high occurrence of pfhA and tbpA genes among *P.multocida* isolates from diseased as well as healthy cattle. Some virulence associated genes such as sodA & sodC were found in a higher percentage among isolates from diseased animals as compared to isolates obtained from apparently healthy animals (Verma et al., 2013). A work carried out by Shivachandra *et al.,*(2013) suggests that a recombinant OmpH (a gene for outer membrane protein H) might be a useful vaccine candidate. Surface exposure and high number of copy makes the OmpA-like proteins favourite candidates for vaccine development (Shivachandra *et al.,* 2013). The recent development of recombinant manipulation systems together with the availability of multiple genome sequences may help to explain the association of these genes with pathological conditions in a given host, as well as helping to elucidate pathogenic mechanisms (Wilkie *et al.,* 2012). Currently recombinant technology is revolutionizing the world of vaccine development.

Protein Bands From Pathogenic Strains

Most of the reported proteins have been identified by their band patterns. Protein band patterns can serve as a guide towards effective vaccine developments. Presently more than 60 different outer membrane proteins (OMP) of different strains have been reported to play a role in pathogenesis and immunity (Hatfaludi *et al.,* 2010). Relationship between electrophoretic pattern and serotype properties of isolates have been reported (wheeler, 2009). One of the most identified proteins is the *PM* toxin which has been fully characterized; it is a monomeric protein with the N-terminal region containing the binding and translocation domain that leads to its endocytosis (Baldwin *et al.,* 2004). Amongst the bands reported were seven polypeptide bands of different molecular weights obtained *P. multocida*, the protein ranges from 36.31 kDa to 104.71 kDa (Numan *et al.,* 2008). Results showed that all the isolates belonged to the same serotype (Numan *et al.,* 2008); the 51.29 and 36.31 kDa bands were thick and were recognized as major bands of *P. multocida* whole strain protein. Other bands obtained were 60.26, 69.18, 77.62, 85.11, and 104.71 kDa (Numan *et al.,* 2008), but according to Sridevi *et al.,* (1999), 13 bands were obtained from *P. multocida* whole cell on SDS-PAGE analysis among which 61.27 kDa and 26 kDa bands were major bands.

Another study have reported 5 bands from *P. multocida,* the bands were 27, 32, 35, 37 and 44 kDa (Johnson *et al.,* 1989). However studies with different strains of *P. multocida* by Lee *et al.,* (1991) suggested that there were no serotype specific band patterns with regard to protein and carbohydrate moieties of avian isolates on SDS-PAGE. This is in line with study of Numan *et al.,* (2008). There are several other works done on protein pattern from *P. multocida* that contributes towards identifying a candidate protein and may guides to cloning the gene that encodes the protein. Dabo *et al.,* (1997) for example, reported eight major outer membrane proteins with two major proteins of 35 kDa and 46 kDa molecular weights by SDS-PAGE of bovine *P.multocida* isolated from cattle and pig sources. Pati *et al.,* (1996) also observed ten polypeptide bands having molecular weights of 88 kDa to 25 kDa, amongst these 44.37 kDa and 30 kDa bands were considered as major immunogens. Eight protein bands (94, 84, 53.5, 49, 43, 41, 29.5 and 16 kDa) on SDS-Page analysis have been reported (Morton *et al.,* 1996). The protein band of molecular weight 43 kDa was present in all serovars.

Several other works (Ireland *et al.,* 1991; Lee *et al.,* 1991; Syuto & Matsumoto, 1982) suggested that the number of protein and carbohydrate bands resolved on SDS-PAGE were different from one study to another and that may be attribued to the type and strain of the antigen.

Studying these proteins and their band patterns as well as comparative analysis may therefore open a new way towards vaccine development especially with announcement of more whole genome sequence of *P. multocida*. It is more than 10 years now since the establishment of more genome sequence result of *P. multocida* (May *et al.,* 2001) however; most of these proteins remain uncharacterized (Boyce et al 2012). The few characterized proteins of outer membrane are OmpH, OmpA, P6-like protein, PlpB, GlpQ, Lpp and Oma78 (Boyce et al. 2006). OmpH is the best characterised among them. It was found to be present in basically all bovine isolates (Dabo *et al.,* 2007). Verma *et al.,* (2013) have suggested that more studies needs to be centered on role of these genes in health and disease and how their expression is or can be influenced and regulated under immunosuppression.

Recent Development On Recombinant Clones Towards Subunit Vaccines

Recent recombinant effort toward recombinant vaccine development by researchers (Table 2) may probably yield positive result for the next generation vaccines. A laboratory study by Hussaini *et al.,* (2011) have shown that a recombinant clone of ABA 392 obtained from *P. multocida* strain *(B:* 2) could have a strong potential for vaccine development. Two immunogenic fussion (OmpH and OmpA) was done recently by Gong et al 2013, and suggested they may have immunogenicity and efficacy of immune protection against fowl cholera. Fusion between OmpA and OmpH vaccine may bring promising result and, provide a valuable reference for the designing of future DNA vaccines for avian *P. multocida* (Gong *et al.,* 2013). Most recent work published by Shivachandra *et al.,* 2014, indicated potential possibilities of using absolutely conserved vacj gene (coding for outer membrane lipoprotein) either as 'signature gene/protein' in developing diagnostic assay or as a recombinant subunit vaccine for *P. multocida* infections in livestock. Filamentous hemagglutinin (FHAB2) is one of the candidate virulent factors, a recombinant protein of FBAH2 derived from *P. multocida* serotype A: 3 and has been shown to have a significant protection against lethal challenge with *P. multocida* (Johnson *et al.,* 2013). A product of *plpB* gene (63kDa) consisting of 831bp has been proven to provide 20–30% protection against *P. multocida* serotype A: 1 and A: 3, 4 infections. The plpB protein may not be an appropriate target as a candidate subunit vaccine for *P. multocida* infection (Chomnawang *et al.,* 2009). It may be possible to have a higher yield of protection of the plpB gene product by fussion with another strong candidate gene. Other researchers are currently paying attention to the use of DNA obtained from outer membrane gene of *P. multocida* as vaccines (Singh *et al.,* 2011; Okay *et al.,* 2012).

Table 2: Recent work on Recombinant Technology for vaccine development against *P. multocida*

Recombinant Clone	Size of gene/Insert (bp)	Size of major protein expressed (kDa)	Sero group/type	Model animal for immunogenicity determination	Protection after direct immunization (%)	Reference
CSI57J (ABA392)	921	26	B:202	Mice	90	Hussain *et al.*, 2012.
OmpH	1002	32	B:2	Mice	N/A	Singh *et al.*, 2011.
tbpA tbpa+IL2 (adjuvant)	2300 2300+500	N/A N/A	B:2	Buffaloes	50 66.6	Shivachandra *et al.*, 2011.
rOmp87	102	87	B:2	Cattle, buffalo, sheep, goat, pig rabbit, chicken, duck, quail, turkey, goose leopard and deer	66.6	Kumar *et al.*, 2014.
plpE + OmpH	474 + 969	35+17	A:3	Mice	40	Okay *et al.*, 2012.
PMT2.3	2300	146	D	Fowl	High level	Lee *et al.*, 2012.
LKTA + PlpE + inactivated bacterin	846 + 1014	30.9 + 38.4	A:1 and A:6	Rabbit and sheep	100	Guzmán-Brambila *et al.*,2012
pPtfA	414	31	B	Pigs, sheep and goats	N/A	Shivachandra *et al.*, 2012.
plpB	831	63	A	Mice	20-30	Chomnawang *et al.*, 2009.
pOMPH + pOMPA	1047 +1050	N/A (transfection)	CVCC474	Chickens	70	Gong *et al.*, 2013.

N/A = Not applicable

The first report of a recombinant *P. multocida* antigen that confers cross protection on animals was reported by Wu *et al.*, 2007, using a recombinant clone of PlpE and PlpB gene from strain of *P. multocida* X-73. Several genes are however yet to be tried as recombinant subunit vaccines. Genes with strong homology or conserved sequences may likely be a good candidate for broad spectrum recombinant vaccine development.

Whole Genome Sequence Of *Pasteurella Multocida*

Success for vaccine development depends largely on the availability of several whole genome sequences of different strains of *P. multocida*. Presently three (3) *P. multocida* species have been fully sequenced (Table 3). It is of importance to mention that the current complete sequenced genome of *P. multocida* ranges between 2 and 2.4Mbp with G+C content of between 40 and 41% (Boyce *et al.*, 2012). The complete pathogenesis of haemorrhagic septicaemia is still an enigma.

Currently, only four complete genome sequences of *P. multocida* subsp. *Multocida* strains are available, those for Pm70 (GenBank accession number AE004439), 3480 (accession number CP001409), HN06 (accession number CP003313), and 36950 (accession number CP003022), which belong to serotypes F, A, D, and A, respectively (Huan *et al.*, 2013). With the subsequent unraveling of more complete genome sequence of *P. multocida*, the exact virulence factor of the pathogenicity of this organism may soon be clear. The elusive determinants of cross-protective immunity still pose a substantial challenge to the scientific community (Shivachandra *et al.*, 2011).

Table 3: Completed whole genome sequence some species of *P. multocida*

PM	Strain	Genomic Size	Reference
HN06	D	2.4Mb	Liu *et al.*, 2012.
PM70	A:3	2.25Mb	May *et al.*, 2001.
36950	A:3	2.35	Michael *et al.*, 2012.

There are other incomplete (Draft) whole genome sequences such as the P2095, AnanD1 and X73 (Johnson et al., 2013) and VP161 , M1404 (B:2) and P903 (D:11), PBA100 A:1, 1059 (Ireland *et al.*, 1991) . The draft genome has recently been reported of PMTB (Huan *et al.*, 2013) with 2.20Mb of serotype B: 2. Even though the pace in the complete whole genome sequence analysis has been very slow, but it is hoped that in the next few years more whole genome sequence of PM will be announced.

CONCLUSION

There is inconsistency with the development of vaccines; this might probably be due to the heterogeneity of *P. multocida*. It is therefore important to develop an efficient

vaccine towards protection against most strains of *P. multocida*. Most of the currently used bacterial vaccines do not confer full protection; as such more effort is required for the development of better vaccines. A possible challenge towards the use of recombinant technology for vaccines development is that the cloned copy of the gene might not fully expressed it proteins as such making the development of the vaccine difficult. Different strains of pathogenic bacteria poses a bigger challenge in vaccine development, as some vaccines may not really be protective against all strains. It should be noted that targeting conserved regions of various strains can be more effective for cross protective vaccines development. Gene expression and characterization of proteins will aid proper diagnosis and antigen determination. Gene expression profiling needs to be carried out on reported candidate or virulence genes to ascertain the pathogenicity to their host. Genome sequencing of more *P. multocida* specie can help to bridge the gap in antigen determination. Single Nucleotide polymorphisms can further be studied of the virulence genes as well as understanding their regulatory and transcriptional factors.

The use of microarray may aid faster diagnosis. Linkage analysis between virulent genes may seem promising in the effort to streamline and identify association of virulent genes in pathogenicity. With the current research trend, it is hope that recombinant vaccine against heterologous species of *P. multocida* may soon become realistic.

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