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Review Article

Progress in DNA Vaccinology against Bacterial Diseases– An Update

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ABSTRACT

DNA vaccines are the emerging promising approach to protect humans and animals from various infections. However, their application to bacterial infections has been scarcely advanced compared to viral DNA vaccines and, till date no licensed bacterial DNA vaccines are available. The various limitations of currently available bacterial vaccines, dangers due to the surge of antibiotic resistant bacteria, a decreased rate of discovery and development of new antibiotics, lack of efficient vaccines against many bacteria and threat of bioterrorism necessitate development of newer technology such as DNA vaccines against bacterial pathogens. Advancements in vector and antigen design, improved formulations and delivery devices/methods, inclusion of adjuvants and prime boosting strategy have greatly enhanced the immunogenicity of many DNA vaccines. This improved performance has spurred a renewed interest in bacterial DNA platform, which is reflected by the numerous ongoing experiments. Because of the strong cell–mediated immunity they can induce, DNA vaccinology is a promising method even against intracellular bacteria. DNA immunization studies have been conducted to combat various significant bacterial diseases such as anthrax, brucellosis, mycobacterial infections, tetanus, leptospirosis, borreliosis, staphylococcosis, mycoplasmosis, caseous lymphadenitis *Pseudomonas aeruginosa* infetctions, *Rhodococcus equi* pneumonia, *Escherichia coli* infection, chlamydiosis, typhoid, yersiniosis, *Helicobacter pylori* infection, streptococcal pneumonia and dental caries. The results of these experiments are quite encouraging which indicates that bacterial DNA vaccines are likely to become a reality in the near future. The present review provides updated information about DNA vaccinology and second generation DNA vaccine optimization strategies against the important bacterial pathogens, along with the concerns and future prospects that will help to improve their potency in order to achieve better outcomes in future clinical trials.

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INTRODUCTION

Immunization indisputably is the most effective intervention in the medical history to control and prevent infectious diseases. A number of dreadful diseases which have previously overwhelmed medical and veterinary field such as small pox, polio, rinderpest and diphtheria, have now become the diseases of past due to the wide spread use of efficient vaccines (Ghanem et al., 2013). However, a number of diseases have not yet been conquered by vaccines and millions of animals, and humans die each year from infectious diseases for which there is no effective vaccine (Curtiss, 2011). Additionally, there are many concerns associated with the use of first generation (vaccines based on attenuated and killed forms of microorganisms) and second generation vaccines (vaccines using defined natural or recombinant protein or proteinaceous components of whole organisms) (Mateen and Irshad, 2011) which are summarized in Table 1. These limitations continue to drive the need for developing novel technologies that offer easier production, administration and better protection without any adverse effects. DNA vaccines based on plasmid offer such an opportunity and are emerging as a promising system against many infectious diseases (Dhama et al., 2008) and such plasmid based vaccines are considered as the third generation vaccines (Tuteja, 1999).

DNA vaccines are basically recombinant bacterial plasmids which normally contain two units: first, the antigen expression unit composed of strong eukaryotic promoter, antigen–encoding gene and transcription termination/polyadenylation sequences to stabilize mRNA transcripts; second, the production unit composed of bacterial origin of replication allowing growth and amplification in bacteria and selectable marker, such as antibiotic resistance gene to facilitate the selection of transformed bacteria (Kumar et al., 2013). The basic steps in the construction of DNA vaccines of the first generation (Jumba, 2010) are shown in Fig. 1. The purpose of the present article is to provide an updated summary on DNA vaccinology against bacterial diseases that help the scientific community to avail all the scattered data in a more concise format.

Background

The concept of DNA vaccine was evolved by Wolf et al. (1990) when they demonstrated that direct transfer of recombinant bacterial plasmid encoding beta galactosidase into mouse muscle induced expression of the protein within muscle cells. Subsequently, Tang et al. (1992) gave the first report that

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introducing a protein encoding gene directly into the skin of mice by propelling DNA–coated gold microprojectiles could elicit antibody responses against the delivered antigen. .

Table 1: Limitations of the first and second generation vaccines

Sr. No.	Limitations	References
A	FIRST GENERATION VACCINES	
1	Killed vaccines	
	1. Immunity induced decreases as time progresses	Ferraro et al., 2011
	2. Requires boosters to achieve life–long immunity	Ferraro et al., 2011
	3. Less likely to induce cell mediated immune (CMI) response	Kindt et al., 2007
	4. Pose risk of infection to those involved in the manufacturing	Kindt et al., 2007
	5. Adjuvants are necessary	Tizard, 2012
2	Live attenuated vaccines	
	1. Pose risk of reversion to pathogenic forms	Tizard, 2012
	2. Maintenance of cold chain is required during storage and transport	Bellet and Prose,2005
	3. Chance of residual virulence in immunized Individuals	Kindt et al., 2007
	4. Chance of containing live contaminating organisms	Tizard, 2012
	5. Not safe in immune–deficient individuals	Kindt et al., 2007
B	SECOND GENERATION VACCINES	
	1. Poorly immunogenic	Tizard, 2012
	2. Less likely to induce CMI response	Tizard, 2012
	3. Preparation and purification of native protein is tedious, costly and pose potential risk	Mateen and Irshad,2011
	4. Proteins produced using heterologous expression system may not be correctly folded	Tizard, 2012
	5. Require adjuvants and booster injections	Tizard, 2012

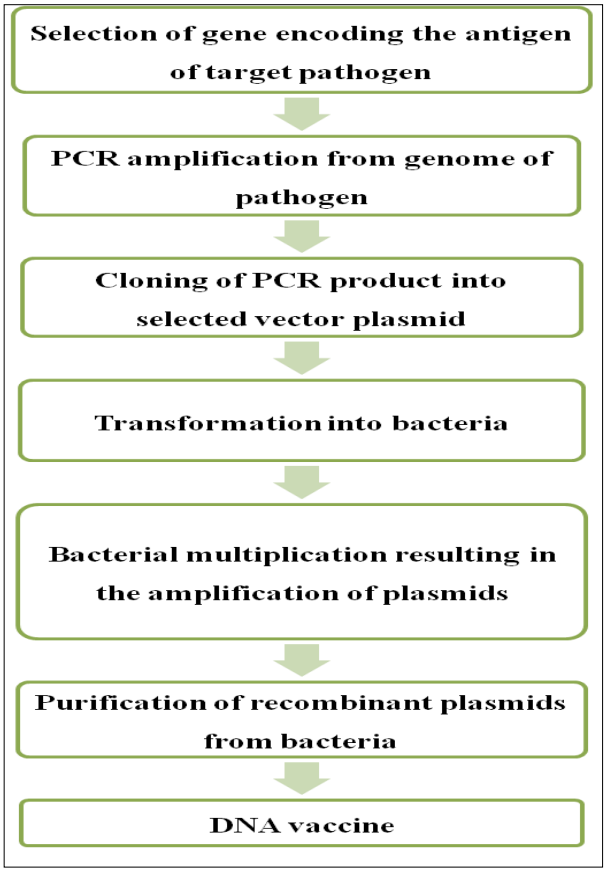

Figure 1: The basic steps in the construction of the first generation DNA vaccines

Table 2: Advantages of DNA immunization strategy

Sr. No.	Advantages	References
1	Easy and fast development and production	Wang et al., 2011
2	Can be repeatedly administered	Wang et al., 2011
3	A highly defined clean vaccine	Kindt et al., 2007
4	Highly stable compared to other biological polymers	Wang et al., 2011
5	Easy to store and use (no need for cold chain maintenance)	Kindt et al., 2007
6	Devoid of any risk of reversion and adverse side–effects	Ferreira et al., 2000
7	Induce both humoral and cellular responses	Wang et al., 2011
8	Can be used as marker vaccines	Donnelly et al., 1997
9	Can be used for therapeutic purpose	Donnelly et al., 1997
10	Since encoded protein is expressed in host it will be exactly similar to the native protein produced in infection	Hechard and Grepinet, 2004
11	Cause prolonged expression of the antigen leading to significant immunological memory	Kindt et al., 2007
12	Bacterial production of plasmid avoids the risk of potential contamination with viruses/proteins present in eukaryotic cell lines used to produce conventional vaccines	Kindt et al., 2007
13	DNA is inexpensive compared to isolated proteins or organisms used for conventional vaccines	Ghanem et al., 2013
14	Can induce immunity even in the presence of high titre of maternal antibody	Tizard, 2012

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One year later, three more papers were published regarding the ability of DNA vectors to drive both humoral and cellular immune responses against pathogens or tumour antigens *in–vivo* (Ulmer et al., 1993; Fynan et al., 1993; Wang et al., 1993). Altogether these reports provided evidence to the scientific community that this simple technique could be developed to create immunity against proteins (Liu, 2011). Afterwards considering the potential advantages of this immunization strategy (Table 2) several experimental trials and safety evaluation of various DNA vaccines have been conducted (Ferraro et al., 2011). Currently, there are 4 licensed DNA vaccines which are against infectious haematopoietic necrosis of salmon in Canada, West Nile fever of horses in USA, melanoma of dogs in USA and DNA vaccine encoding growth hormone releasing hormone for swine in Australia (Williams, 2013)

Mechanism of Protection By DNA Vaccines

When a DNA vaccine is injected into the host it enters host cells (transfection) and antigenic protein is expressed endogenously. There are three possibilities for the transfection such as direct transfection of antigen presenting cells especially Dendritic Cells (DCs), direct transfection of somatic cells and cross–priming. Antigen presentation mediated by MHC–I and MHC–II pathways is then followed (Gurunathan et al., 2000; Dunham, 2002) resulting in the induction of both cellular and humoral immune response against the antigen (Shedlock and Weiner, 2000). So, DNA vaccines are considered to be the best tool to prevent various infectious diseases especially against intracellular pathogens (Moreno and Timon, 2004). In addition to the antigen, unmethylated CpG motifs present in the plasmid backbone that are recognized by TLR9 can also cause stimulation of DCs which in turn promote strong cell mediated immune response (Klinman et al., 1997).

Second Generation DNA Vaccines

Early in the clinical trials of DNA vaccines it was found that these are well–tolerated & safe vaccines without induction of autoimmunity and tolerance. There was both humoral and cell mediated immune responses but, the potency was found to be disappointing (Liu, 2003). As a result varieties of approaches are now under evaluation to increase the potency of DNA vaccine whilst still retaining their attractive features. These optimization strategies (Fig. 2; Table 3) lead to the development of second generation DNA vaccines (Donnelly et al., 2005; Kutzler and Weiner, 2008).

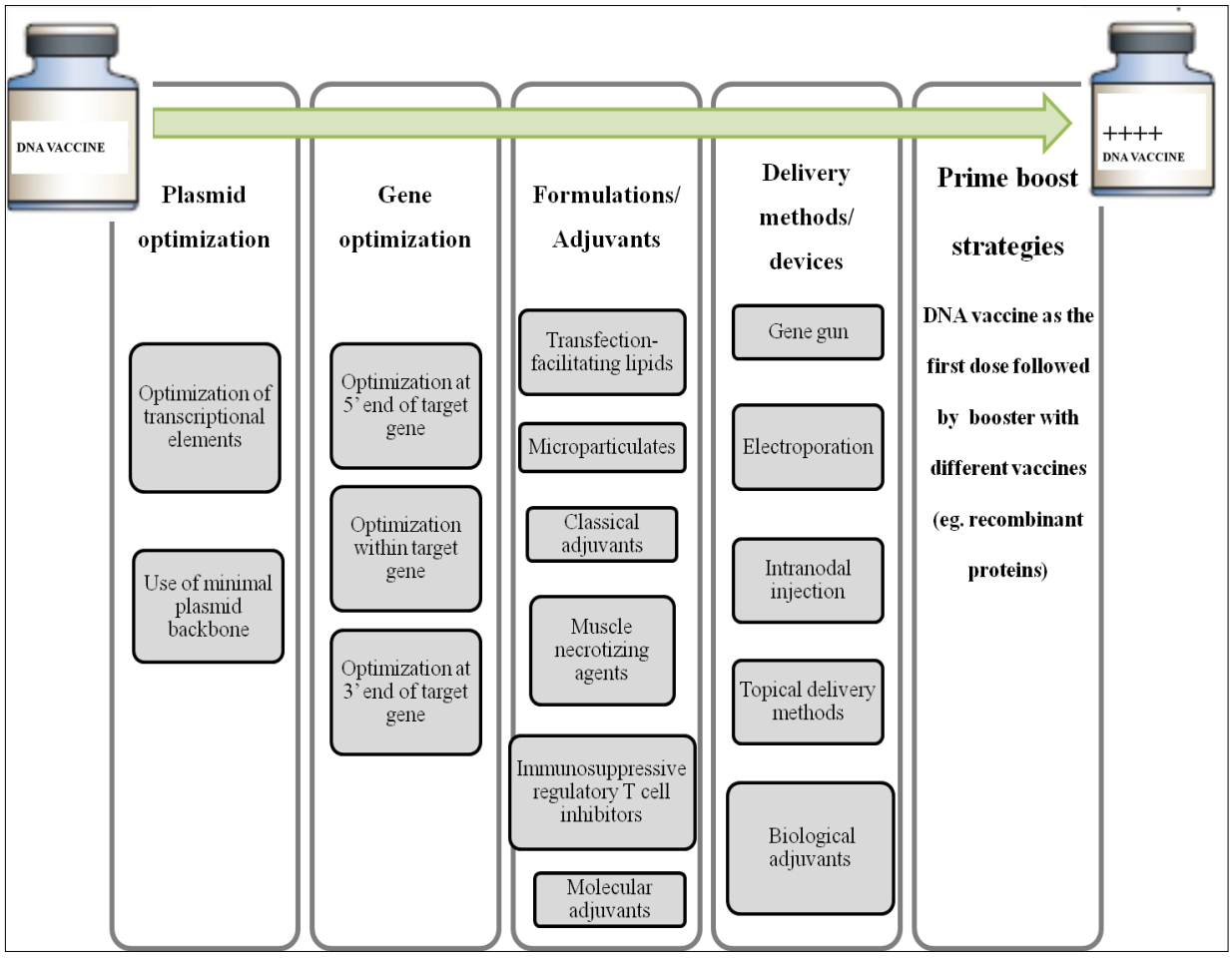

Figure 2: The optimization strategies for second generation DNA vaccines

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Table 3: Optimization strategies for improving the potency of DNA vaccines

Optimization strategy	Strategy
Optimization of transcriptional elements	Use of stronger and appropriate eukaryotic promoters, inclusion of proper termination site, inclusion of transcriptional transactivators/ enhancer elements
Optimization at 5' end of target gene (Gene encoding antigen)	Modification or insertion of Kozak consensus sequence upstream of the gene, removal of sequences responsible for lower expression, addition or modification of leader sequences, facilitating cell surface expression or secretion by inserting secretory signal sequences, inclusion of synthetic hairpin oligonucleotides
Optimization within the target gene	Codon optimization according the species, mRNA optimization to improve the stability, expression of multiple short antigenic peptides joined together in a "string of beads" approach, elimination of glycosylation sites, use of consensus immunogen, fusion to a pathogen sequence, such as fragment C of tetanus toxin or to ligands for APC receptors or to proteins capable of intercellular transport or to molecules capable of binding to DCs
Optimization at 3' end of target gene	Addition of double stop codon, inclusion of synthetic hairpin oligonucleotides
Transfection facilitating lipid complexes	Varying combinations of cationic lipids and cholesterol
Microparticulates	DNA adsorbed to or entrapped in biodegradable microparticles such as polylactide coglycolide or chitosan, or complexed with non–ionic block copolymers or polycations such as polyethyleneimine
Classical adjuvants	Aluminium phosphates, Saponin
Agents that causes muscle necrosis during i/m delivery	Cardiotoxin or bupivicaine, injection of relatively larger volumes of fluid
Agents that eliminates immunosuppressive regulatory T cells	Cyclophosphamide, fludarabine, IL–2 immunotoxin, and COX–2 inhibitors
Molecular adjuvants	Plasmid encoding cytokines, chemokines, co–stimulatory molecules, toll like receptor ligands, small interfering RNA targeting immunosuppressive factors, oligodeoxynucleotides containing unmethylated CpG motifs or anti–apoptotic proteins of DCs as part of the plasmid vaccines cocktail
Topical delivery methods	Transcutaneous microneedles, use of iontophoresis or sonophoresis as potent physical adjuvants for transcutaneous immunization, dermal patch, mucosal jet injector, tattoo perforating needle, painting DNA with multiple administrations of adhesive tape in combination with sodium dodecyl sulphate or urea cream (or both)
Biological adjuvants	Use of live–attenuated intracellular bacteria as delivery systems such as *Salmonella* Typhi, *Listeria monocytogenes*, *Shigella flexneri*, *Yersinia enterocolitica* or invasive *E. coli*, use of any aother biological products as adjuvants

Progress in DNA Vaccinology against Bacterial Diseases

There are significant advances in the development of DNA vaccines against viral pathogens, while their application to bacterial infections has been scarcely advanced and till to date no licensed bacterial DNA vaccines are available. The reasons for the less advancement in DNA vaccinology against bacterial diseases may be the successful performance of many conventional vaccines and antibiotics against bacterial infections. However, the clear cut advantages of DNA vaccines over other vaccines (Table 2), the dangers due to the surge of antibiotic resistant bacteria, lack of efficient vaccines against many bacteria and the threat of bioterrorism necessitate development of DNA vaccines against bacterial pathogens. Moreover, DNA vaccination is a promising method o fight against intracellular bacteria since it can efficiently induce a cellular immune response (Nagata and Koide, 2010). As complete genome sequences of many bacteria have been recently decoded, researchers can now select the appropriate antigens and design the specific DNA vaccine construct (Ingolotti et al., 2010). Although there are some specific concerns in developing DNA constructs against bacteria such as difficulties in folding, transport and post–translational modifications of prokaryotic proteins in eukayotic cells leading to unwanted effects after immunization (Reyes–Sandoval and Ertl, 2001) and limitation in designing DNA construct against capsular polysachharide antigens (Tizard, 2012), DNA vaccine technology has been applied against different types of bacteria which can be summarized as follows

Bacillus anthracis

B. anthracis is a spore–forming, Gram–positive bacterium that causes a fulminating disease called anthrax in mammalian livestock and humans. The currently available anthrax vaccines in both animals and human are far from ideal. The need for a well defined vaccine that can stimulate both humoral (essential for toxin neutralization and protection) and cellular arms of adaptive immunity (for the clearance of encapsulated *B. anthracis*) (Glomski et al., 2007) and that can be prepared without handling the dangerous pathogen and increasing threat of bioterrorism *etc* drive the scientists to develop DNA vaccine against anthrax. The work towards anti–anthrax DNA vaccine mainly included demonstration of anti–PA immune response in mice, rats and rabbits (Gu et al., 1999; Luxembourg et al., 2008). However, reports of successful protection of hosts through the administration of PA–encoding DNA alone have been limited (Riemenschneider et al., 2003; Midha and Bhatnagar, 2009). Therefore, some investigators (Price et al., 2001b; Hahn et al., 2006; Zhang et al., 2008) later on tried DNA constructs encoding other vaccine candidates of anthrax bacilli (lethal factor, spore antigens or vegetative cell antigens) along with PA encoding construct and found that co–administration of these vaccine candidates could generate synergestic effect. In the attempt to further enhance the intensity of immune response to *B. anthracis* several groups have attempted many second generation DNA vaccination strategies which are depicted in Table 4).

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Table 4: Optimization strategies used in second generation bacterial DNA vaccines

Sr. No.	Bacteria/ Disease	Optimization strategy used	References
1	*B. anthracis*	DNA prime/protein boost strategy	Williamson et al., 1999; Price et al., 2001b
		Secretory DNA vaccine construct	Galloway and Baillie, 2004
		Recombinant adeno associated virus vector based formulation	Liu et al., 2009
		Electroporation	Luxembourg et al., 2008
		Cationic lipid–formulation	Hermanson et al., 2004
2	*B. abortus*	Escheriosomes formulation	Singha et al., 2008
		Y. enterocolitica as vehicle	Al–Mariri et al., 2002
		Intraspleen delivery	Munoz–Montesino et al., 2004
3	*M. tuberculosis*	Molecular adjuvant (IL–2)	Wang et al., 2008; Changhong et al., 2009; Okada et al., 2009; Hanif et al., 2010
		Autophagy inducing system + chitosan based formulation	Meerak et al., 2013
		DNA prime/protein boost strategy	Jiang et al., 2013
		Gene gun	Nagata and Koide, 2013
		Cationic lipid–formulation	D'Souza et al., 2002; Rosada et al., 2008
		Modification of leader sequence	Malin et al., 2000
		Biological adjuvant	Brun et al., 2008
4	*C. tetani*	Codon optimization	Stratford et al., 2000
5	*C. abortus*	DNA prime/protein boost strategy	Coulter et al., 2002
		Gene gun	Hechard et al., 2003
		Inclusion of leader sequence and Kozak modification	Dong– Ji et al., 2000
		Phage mediated delivery	Ling et al., 2011; Ou et al., 2013
6	*C. muridarum*	DNA prime/protein boost strategy	Brown et al., 2012
7	*C. psittaci*	Gene gun	Vanrompay et al., 1999
8	*C. trachomatis*	DNA prime/protein boost strategy	Dong–Ji et al., 2000
9	*B. burgdorferi*	Optimization within target gene by fusion onto human tissue plasminogen activator leader sequence	Scheiblhofer et al., 2003
10	*S. aureus*	Molecular adjuvant (bovine IL18)	Yin et al., 2009
11	*M. pulmonis*	Gene gun	Lai et al., 1995
12	*M. hyopneumoniae*	DNA prime/protein boost strategy	Chen et al., 2006
13	*M. pneumoniae*	Molecular adjuvant (IL2)	Zhu et al., 2013
		Molecular adjuvant (B subunit of *E. coli* heat–labile enterotoxin)	Zhu et al. 2012
		Molecular adjuvant (cholera toxin B)	Han and Dao, 2007
14	*Dental caries*	Gene optimization by fusion to cytotoxic T lymphocyte antigen–4 (CTLA4) for APC targeting	Xu et al., 2005; Jia et al., 2006
		Biological adjuvant (Recombinant flagellin (FliC) from Salmonella	Shi et al. 2012
15	*P. aeuriginosa*	Biological adjuvant (synthetic peptide–keyhole limpet hemocyanin conjugate or chimeric influenza virus)	Price et al., 2002
16	*R. equi*	Molecular adjuvant (IL12)	Haghighi and Prescott, 2005
		Optimization within target gene by fusion onto human tissue plasminogen activator leader sequence	Wang et al., 2004a
17	*Y. pestis*	Molecular adjuvant (IL12)	Yamanaka et al. 2008; 2009
		DNA prime/protein boost strategy, use of stronger and appropriate eukaryotic promoters, gene gun	Garmory et al., 2004
		Gene gun	Bennett et al. 1999
18	*H. pylori*	Molecular adjuvant (IL–2 and B subunit heat–labile toxin of *E. coli*)	Chen et al., 2012
		Molecular adjuvant (IL–2)	Yu et al., 2010

Brucellosis

Brucellosis, an infectious disease affecting livestock and humans by different species of *Brucella* remains endemic in many developing countries, where it undermines animal health and productivity, causing important economic losses. In human it is a potentially life–threatening multisystem disease. Unfortunately, the current vaccines are not ideal because of their limited efficacy and potential to cause disease in humans (Singha et al., 2008). Consequently, numerous attempts are made to develop efficient DNA vaccines against brucellosis which are briefly outlined below.

It was Kurar and Splitter (1997) who started DNA based immunization studies of brucellosis. They used plasmid expressing ribosomal protein L7/L12, to immunize mice and found that the protection induced after 28 days of immunization against challenge was equivalent to that induced

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by live *B. abortus* strain 19 vaccine. The further DNA vaccination trials of *B. abortus* included immune response studies against plasmid encoding Cu, Zn superoxide dismutase (Onate *et al*., 2003; Singha et al., 2008), plasmid encoding GroEL heat–shock gene (Leclero et al., 2002), plasmid encoding bacterioferritin or P39 gene (Al–Mariri et al., 2001), divalent fusion DNA vaccine encoding L7/L12 and Omp16 (Luo et al., 2006) and a combined DNA vaccine encoding three antigens BCSP31, SOD, and L7/L12 (Yu *et al*., 2007). Some second generation DNA vaccine optimization strategies have also been attempted (Table 4). In case of *B. melitensis*, DNA vaccine encoding *omp31* (Cassattaro et al., 2005; Doosti et al., 2009) and DNA vaccine encoding *bp26* and trigger factor (Yang et al., 2005) had been tried. Of these DNA vaccine encoding bp26 and Tf was also found to induce immune response in bison (Clapp et al., 2011).

Mycobacterial Infections:

The increasing number of infection cases and lower efficacy of BCG vaccine in controlling pulmonary infection, emergence of multidrug–resistant strains and co–infection with HIV–1 are the major impetus for developing novel vaccine strategy such as DNA vaccine against Mycobacterium (Nagata and Koide, 2013). Because of the strong cell–mediated and humoral immunity they can induce, DNA vaccines were rapidly considered for use against Mycobacterium which are facultative intracellular pathogens and a considerable number of preclinical studies on the subject have been published in recent years (Table 5).In 1996, Tascon et al. and Huygen et al. were the first to report on the value of naked DNA vaccination against TB after using DNA vaccine encoding 65–kDa heat shock protein from *M. leprae* and 32–kDa mycolyl transferase or Ag85A from *M. tuberculosis*, respectively. Afterwards large numbers of studies were conducted and several these DNA vaccines conferred significant protection against TB in mice.

Table 5: Antigens targeted in Mycobacterial DNA vaccines

Sr. No.	Antigens	References
1	6–kDa ESAT–6 (*M. tuberculosis*)/ *M. tb*	Lozes et al., 1997; Li et al., 1999; Lowrie et al., 1999; Fan et al., 2007
2	8.4 kDa (*M. tb*)	Coler et al., 1998
3	17–kDa MPT63 (*M. tb*)	Manca et al., 1997; Li et al., 1999; Pan et al., 2003
4	19–kDa lipoprotein (*M. tb*)	Erb et al.,1998; Yeremeev et al., 2000
5	22–kDa Lppx lipoprotein *(M bovis)*	Lefevre et al., 2000
6	23–kDa MPB83 *(M bovis)*	Chambers et al., 2000; 2002; Vordermeier, 2001
7	24–kDa alkyl hydroperoxide reductase C (*M. tb*)	Erb et al., 1998
8	26–kDa MPT64 (*M. tb*)	Kamath, et al., 1999; Li et al., 1999
9	30–kDa mycolyl transferase Ag85B (*M. tb*)	Kamath, et al., 1999; Roche et al., 2001; Velaz–Faircloth et al., 1999
10	32–kDa mycolyl transferase Ag85A (*M. tb*)	Huygen et al.,1996; Baldwin et al., 1998; Tanghe et al., 2001; Chambers et al., 2002;VelazFaircloth et al., 1999
11	35–kDa antigen (*M. avium*)	Martin et al., 2000
12	35–kDa antigen (*M. leprae*)	Martin et al., 2001
13	36–kDa proline–rich antigen PRA(*M. tb*)	Tascon et al., 1996
14	38–kDa lipoprotein PstS–1(*M. tb*)	Zhu et al., 1997; Tanghe et al., 1999
15	38–kDa lipoprotein PstS–2 (*M.bovis*)	Tanghe et al., 1999
16	40–kDa lipoprotein PstS–3 (*M.bovis*)	Tanghe et al., 1999
17	43 kDa 1818PE–PGRS protein (*M. tb*)	Delogu and Brennan, 2001
18	39 kDa MTB39 protein from PPE family (*M. tb*)	Dillon et al., 1999
19	65 kDa heat shock protein (*M. leprae*)	Tascon et al., 1996
20	65 kDa heat shock protein (*M. tb*)	Turner et al., 2000
21	65 kDa heat shock protein (*M. avium*)	Velaz–Faircloth et al., 1999
22	70 kDa heat shock protein (*M. tb*)	Lowrie et al., 1997
23	84 kDa KatG (*M. tb*)	Li et al., 1999
24	Ag85B, MPT64,MPT83 (*M. tb*)	Cai et al., 2005
25	Fbp/Htpx (*M. tb*)	Brun et al., 2008
26	Mtb72F (*M. tb*)	Reed et al., 2009
27	Rv3407 (*M. tb*)	Mollenkopf et al., 2004
28	Hsp65 (*M. tb*)	Okada et al., 2009
29	MPT64/MPT83 (*M. tb*)	Tian et al., 2005
30	RD1 PE35, PPE68, EsxA, EsxB, RD9 and EsxV (*M. tb*)	Hanif et al., 2010
31	HSP–65 (*M. paratuberculosis*)	Sechi et al.,2006
32	Rv3407, Ag85A and HspX (*M.tb*)	Mir et al., 2009
33	Ag85B (*M. bovis*)	Teixeira et al., 2006
34	ESAT–6 and FL (fms–like tyrosine kinase 3 ligand) (*M. tb*)	Jiang et al., 2013
35	ESAT–6, MPT–64, MPT–83, and KatG (*M. tb*)	Morris et al., 2000

Although to date none of the vaccines have been assessed in human results of trials in rodents have demonstrated the potential of mycobacterial DNA vaccines in larger animals and humans. Also, immunization protocols combining the optimization strategies for second generation DNA vaccines especially potent priming capacity of plasmid DNA with

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subsequent boosting with BCG are particularly promising for future applications (Jiang et al., 2013)

Clostridium tetani

Anderson et al. (1996) used plasmid encoding the non–toxic C–terminal domain of tetanus toxin to immunize BALB/c mice and found that DNA immunization induced a Th1–like response. In contrast, immunization with tetanus toxoid or a polypeptide of fragment C induced a Th2–like response. The serum immunoglobulin response following DNA immunization was sufficient to protect 100% of mice from lethal challenge with tetanus toxin. However, the level of protection conferred by DNA immunization was lesser than that achieved with conventional toxoid or a polypeptide of fragment C. The results of Saikh et al. (1998) also suggested that polypeptide or toxoid vaccines are preferable to plasmid–based vaccination for control of tetanus so that there were no further studies in DNA vaccination against tetanus

Leptospirosis

Leptospirosis, a zoonosis caused by bacteria of the genus Leptospira, is an important emerging infectious disease worldwide. Available leptospirosis vaccines made up of inactivated bacteria or their membrane components elicit only serovar specific immunity and unsatisfactory immunological memory. The advantages of DNA vaccines over the inactivated vaccines and requirement of a broad spectrum anti–leptospira vaccine that induces long–lasting memory led to the development of different DNA constructs against leptospirosis. Dai (1998) reported that DNA vaccination with the sero–reactive P68 antigen protected 77% of vaccinated guinea pigs from death and 85% from pulmonary diffuse hemorrhage, following challenge with *L. interrogans*. Another study with DNA vaccine expressing Hap–1/LipL32 of *L. interrogans* serovar Autumnalis or Grippotyphosa, used in gerbils showed cross–protection against challenge with Canicola (Branger et al., 2005). Similarly, many works (You et al., 1999; Dai et al., 2000; Wang et al., 2002; Dai et al. 2003) has suggested that DNA vaccines based on endoflagellar (*flaB2*) gene can be successful vaccines against Leptospira as CpG motif found within the gene could give additional immune–stimulatory property. Likewise, Faisal et al. (2008) showed that immunization with LigA DNA vaccine could provide significant protection against challenge with virulent *L. interrogans* serovar Pomona. Very recently, Forster et al. (2013) showed that LigBrep DNA vaccine is a promising candidate against leptospirosis. All these observations suggest that use of DNA constructs encoding various immunogenic leptospiral proteins can be a promising approach for protection against leptospirosis.

Borrelia burgdorferi

Although lyme borreliosis caused by *Borrelia* spp. is the most prevalent arthropod–borne disease in the Western world, no vaccines are currently available to prevent the disease (Yin et al., 2009). The role of outer surface protein genes (*ospA* and *ospC*) of *B. burgdorferi* to elicit protective immune responses when administered as DNA vaccines has been explored (Luke et al., 1997; Wallich et al., 2001; Scheiblhofer et al., 2003), the results of which shows that DNA vaccines against borreliosis need more extensive investigations.

Staphylococcus aureus

S. aureus is one of the five most common causes of nosocomial infections and can cause a range of illnesses from minor skin infections to many life–threatening diseases. Ohwada et al. (1999) used DNA encoding *mecA* to immunize mice against methicillin–resistant *S. aureus* infection and showed that the vaccination could produce 0.4 log reduction in kidney CFU compared to control animals. Similarly, Senna et al. (2003) showed that vaccination of mice with plasmid encoding PBP2a protein could reduce the bacterial load of kidneys 1000 times less compared to the non–immunized mice. DNA vaccine comprising of *clfA* or fibronectin binding protein gene was also shown to induce sufficient protection against *S. aureus* infection (Shkreta et al., 2004; Nour El–Din et al., 2006). Castagliuolo et al. (2006) showed that intranasal immunization with a DNA vaccine mixture encoding four adhesins of *S. aureus* (fibrinogen binding protein Efb, fibronectin–binding protein A (FnbpA), clumping factor A (ClfA) and collagen–binding protein (Cna) could trigger significant levels of specific serum and mucosal Ig that inhibited *S. aureus* adhesion to cow mammary gland epithelial cells *in–vitro*. Later, Gaudreau et al. 2007) showed that multi gene plasmids (encoding Clfa, FnbpA and the enzyme Sortase) could induce better immune response compared to mixture of the individual plasmids. Very recently, Dai et al. (2013) demonstrated that novel DNA vaccine encoding *M. tuberculosis* secreted antigen Ag85A fused with influenza A virus HA2 protein could provide protection against both influenza and secondary infection with *S. aureus*. Thus, DNA immunization against the most important virulence factors, adhesins of *S. aureus* has been proved as valuable tool to prevent infections in lab animals warranting further studies in target animals.

Mycoplasmosis

Mycoplasma, the smallest self–replicating life–forms are responsible for a variety of diseases in humans, domestic animals, insects, and plants. The various antigens targeted in DNA vaccine studies against Mycoplasmoses included a repeat region of P97 adhesin (Chen et al., 2006), heat shock protein (P42) (Chen et al., 2003), P36, P46, NrdF, P97 and P97R1 (Chen et al., 2008) of *M. hyopneumoniae*, carboxy terminal region of p1 gene of *M. pneumoniae* (Zhu et al., 2012; 2013) and P48 of *M. agalactiae* (Chessa et al., 2008). Very recently, Galli et al. (2012) proved that P46 is a promising candidate for DNA vaccine against *M. hyopneumoniae*. It was also shown that the licensed DNA vaccine encoding growth hormone delivered before specific vaccination could enhance protection against *M. hyopneumoniae* (Thacker et al., 2006).

Corynebacterium pseudotuberculosis

C. pseudotuberculosis, a facultative intracellular bacterium, is the etiological agent of caseous lymphadenitis which is a chronic and contagious disease of sheep and goats worldwide. Although various strategies have been tested to develop vaccine against *C. pseudotuberculosis*, the search continues for identification of an effective and safe vaccine. Chaplin et al. (1999) vaccinated sheep with DNA encoding genetically detoxified phospholipase D, and obtained good protection against experimental challenge. When Costa et al. (2011) immunized mice with DNA vaccine encoding *hsp60* of *C. pseudotuberculosis*, there was significant humoral immune response but immunization did not confer protective immunity. So improvement of the DNA construct of Chaplin et al. (1999) by adopting second generation DNA vaccine optimization strategies or new vaccines encoding alternative antigens should be targeted in future trials.

Pseudomonas aeruginosa

P. aeruginosa was identified as the fifth most frequently isolated nosocomial pathogen and the second most common cause of nosocomial pneumonia. The increasing numbers of antibiotic resistant *P. aeruginosa* further necessitate the need to develop suitable immunization strategies against this pathogen. In a study evaluating the immunoprotective potential of a DNA vaccine encoding *oprF* of *P. aeruginosa* in mouse model of chronic pulmonary infection Price et al. (2001a) found that there was a significant reductions in the presence of severe macroscopic lesions, as well as in the number of bacteria present in the

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lungs, of immunized mice. DNA vaccine encoding a fusion protein comprising *oprF* and another outer membrane protein OprL was later found to be more protective (Price et al., 2002). Denis–Mize et al. (2000) and Shiau (2000) found that upon immunization of mice with DNA encoding the coding sequence of non–toxic mutant form of *P. aeruginosa* exotoxin A there was a strong serum immunoglobulin response and vaccinated mice were completely protected from the lethal effect of intraperitoneal injection with wild–type Exotoxin A. Saha et al. (2006) showed that immunization with DNA vaccine targeting a fusion of outer membrane proteins (OprF/OprI), a protein regulating type III secretion system (PcrV), or an appendage (PilA) produced the strongest immune response and protection against pulmonary infection caused by *P. aeruginosa.*

Rhodococcus equi

R. equi remains as significant bacterial pathogen causing severe pyogranulomatous pneumonia in foals. There is no effective vaccine currently available for the prevention of *R. equi* pneumonia in which the protective immunity is largely based on cell–mediated immune response. DNA vaccine encoding VapA virulence protein of R. *equi* has been found to be able to induce specific IgG antibody response and Th–1 response in foals (Vanniasinkam et al., 2005). Subsequently, Phumoonna et al. (2008) demonstrated that a chimeric vapA/groEL2 DNA vaccine enhanced the clearance of *R. equi* in aerosol challenged mice.

Escherichia coli

Infection with *E. coli* O157:H7 causes bloody diarrhea and hemolytic uremic syndrome with renal failure that can be deadly dangerous. The search for an effective vaccine also includes some immunization trials with different DNA constructs. Caprioli et al. (2005) described anti–Stx2 DNA vaccines encoding either the B subunit or a fusion protein between the B subunit and the first N–terminal amino acid of the A1 subunit of shiga toxin elicited Stx–specific immune responses. Later, Bentancor et al. (2009) developed DNA construct encoding both Stx2 A2 and B subunit and found that this could induce specific humoral responses and could confer *in–vitro* as well as *in–vivo* Stx2 neutralization activity. Recently Shariati et al. (2012) used a triplet synthetic gene (*eit*) designed from three genes (*espA*, *eae* and *tir*) and found that this DNA vaccine could induce protective immunity either alone or in combination with purified antigens to reduce EHEC infection. DNA vaccines against other *E. coli* such as DNA vaccine encoding K88 fimbrial protein (Cho et al., 2004), CFA/I fimbrial adhesin (Alves et al., 1998), *faeG* adhesin gene (Turnes et al. 1999) of enterotoxigenic *E. coli* and adhesin of enteroaggregative *E. coli* (Bouzari et al. 2010) were also reported to elicit satisfactory protection.

Chlamydiosis

They are obligate, intracellular, Gram–negative bacteria that can produce a variety of diseases in humans and animals. As DNA immunization can induce both humoral and cellular immune responses which are especially suited to fight against intracellular bacteria these represent an opportunity for researchers to explore a novel method of vaccination against these pathogens (Ling et al., 2011). The genes tested included MOMP, pgp3, ORF–5 for *C. trachomatis* (Donati et al., 2003; Li et al., 2008), MOMP, variable domains of MOMP and CTP synthetase for *C. muridarum* (Zhang et al., 1997; 1999; Pal et al., 1999; Dongji et al., 2000), Omp2, panel of ORF, Hsp60, MOMP for *C. pneumoniae* (Svanholm et al., 2000; Penttilä et al., 2001), MOMP for *C. psittaci* (Vanrompay et al., 1999) and DnaK (Hsp70) and ompA for *C. abortus* (Hechard et al., 2002; 2003; Ou et al., 2013). Of these MOMP was proved as the most important antigen, however, use of additional chlamydial antigen genes and evaluation of different optimization strategies is necessary in future trials to improve the degree of protection.

Salmonella Typhi

Despite advances in technology and public health strategies, typhoid fever remains as a major cause of morbidity in the developing countries. Surprisingly, there was only one report of DNA vaccination against this disease. When Lopez–Macias et al. (1995) immunized BALB/c mice with DNA expressing the Outer Membrane Protein C Porin of *Salmonell*a Typhi there was a serum IgG response specific to the protein.

Yersiniosis

DNA immunization trials have been attempted against two *Yersinia* Spp namely *Y. pestis* and *Y. enterocolitica.* The maximum trials were against pneumonic plague, a highly lethal and contagious disease caused by *Y. pestis*. DNA vaccine encoding V antigen (Bennett et al., 1999; Wang et al., 2004a; Garmory et al., 2004), F1 capsular antigen (Grosfeld et al., 2003) or both (Yamanaka et al., 2008; 2009) were shown to be protective against pneumonic plague. In case of *Y. enterocolitica* Noll et al. (1999) successfully immunized mice with HSP60 DNA vaccine which were then found to be protected from challenge with lethal dose of *Y. enterocolitica* administered either intravenously or orally.

Helicobacter pylori

H. pylori a Gram–negative microaerophilic spirochete classified as a class I carcinogen, has infected half of the world's human population. The high prevalence of infection, emergence of antibiotic resistant strains, its role in pathogenesis of gastritis, peptic ulcer, MALTomas and adenocarcinomas and difficulty and high cost of treatment make it an important target for DNA vaccination. Different DNA immunization trials using heat shock Protein A or B (Todoroki et al., 2000), urease B (Hatzifoti et al., 2006; Sun et al., 2006; Xu et al., 2007) outer inflammatory protein A (Chen et al., 2012) and Lpp20 antigen (Yu et al. 2010) of *H. pylori* demonstrated that DNA immunization can be as a productive and economic novel method against *H. pylori* in humans.

Streptococcosis

S. pneumoniae is a leading cause of morbidity and mortality in both developing and developed countries. The disadvantages of current polysaccharide or licensed conjugate vaccines and requirement of cell mediated immunity for protection directed the scientists to focus on DNA vaccine against *S. pneumoniae.* Lesinskia et al. (2001) demonstrated that DNA vaccine encoding peptide mimic of *S. pneumoniae* serotype–4 capsular polysaccharide could induce specific anti–carbohydrate antibodies in Balb/c mice. In the same year Miyaji et al. showed that PsaA (pneumococcal surface adhesin A) and PspA (pneumococcal surface protein A) DNA vaccines could induce humoral and cellular immune responses against *S. pneumoniae.* Subsequently, Ferreira et al. (2006, 2010) reported that immunization with a plasmid expressing PspA could protect mice from lethal pneumococcal septicaemia. Another Streptococcus, *S. iniae* is an important fish pathogen with a broad host range that includes both marine and freshwater fish species. Very recently, Sun et al. (2012) demonstrated that DNA vaccines based on sagF, G, and I, especially when they are formulated as multivalent vaccines, were highly efficacious against *S. iniae* infection.

Dental caries is a widespread infectious disease, of which the principal causative agent is *S. mutans*. The production of a safe and cost–effective dental caries vaccine which can block tooth colonization and plaque buildup by *S. mutans* has been a high priority in dental research (Shi et al., 2012). Fan et al.

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(2002) showed that DNA vaccine encoding cell–surface protein antigen (PAc) of *S. mutans* could induce protective anti–caries immune responses. Then, Jia et al. (2006) constructed a fusion anti–caries DNA vaccine encoding GLU fragment of *S. mutans gtfB* gene and A–P fragment of PAc gene and found that this vaccine reduced the levels of dental caries caused by *S. mutans* in gnotobiotic animals. However, the protective effect against *S. sobrinus* infection was weaker (Xu et al., 2007). Thereafter, Niu et al. (2009) showed that addition of DNA vaccine encoding catalytic fragment of *S. sobrinus gtf–I* gene to the above vaccine could provide better protection against caries. Some second generation DNA vaccine optimization strategies has also been tested against dental caries (Table 4) Thus, the idea of anti dental caries DNA vaccine seems to be attractive, but the full potential of DNA vaccines has not yet been fully realized which has to be achieved in future studies.

Concerns and Future Prospects

Despite the numerous advantages some issues have been raised with regard to DNA vaccines. First, integration of injected vaccine DNA might occur in the genome of the host cell which may result in insertional mutagenesis, activation of oncogenes or inactivation of tumor suppressor genes (Martin et al., 1999). Second, repeated injections can induce autoimmunity (Mor et al., 1997). Finally, the antibiotic resistance gene carried on vector plasmid can introduce that resistance property in immunized animals or humans when that plasmid is used for vaccination. However, exhaustive research has found little evidence of integration, and the risk for integration appears to be significantly lower than that associated with naturally occurring mutations (Ledwith et al., 2000; Wang et al., 2004b; Sheets et al., 2006). Studies have also shown that evidence for the changes in clinical markers of autoimmunity have not been reported and early human studies did not detect any increase in anti–DNA antibodies, therefore, systemic autoimmunity is unlikely to result from DNA vaccination (MacGregor et al., 1998; Tavel et al., 2007; Klug et al., 2012). For avoiding the antibiotic resistance two precautions should be kept during the selection of vector plasmid. First, the antibiotic resistance genes contained by vaccine plasmids are driven by bacterial origin of replication (not mammalian one) and are therefore expressed only in bacteria, not in host cells. Second, the antibiotic resistance employed does not involve antibiotics commonly used to treat human infections (Mateen and Irshad, 2011). Overall, multiple studies have reported that DNA platform is well tolerated and have an enviable safety record.

DNA vaccines are emerging as a promising new approach to protect humans and animals from various infections (Dhama et al., 2008; Nagata and Koide, 2010; Liu et al., 2011; Ghanem et al., 2013; Kumar et al., 2013). However, their application to bacterial infections has been scarcely advanced compared to viral DNA vaccines and, till to date no licensed bacterial DNA vaccines are available. On the other hand, a great deal of progress has been made in bacterial DNA vaccinology which indicates that these are likely to become a reality in the nearby future. The significant obstacle to the successful development of DNA vaccines has been the low efficacy in the induction of immune response. The recent sequencing of the complete genomes of many pathogenic bacteria will help in the identification of novel antigen candidates for DNA vaccination (Ingolotti et al., 2010). Another potentially interesting study would be to implement an expression library immunization and to screen it *in vitro*, as well as *in vivo*, for its protective effect. Such a novel approach will be very useful for the rapid identification of protective genes especially for microorganisms that are difficult to grow or attenuate, such as Chlamydiaceae (Leclercq et al., 2003).

It is now well known that DNA vaccination efficiency also depends on delivery method, dose of immunization and challenge, adjuvants as well as the species and strain of the animal used for immunization study. Therefore, as the protective effect of a given antigen can differ depending on the immunization protocol, it seems important to first test the same antigen with different immunization protocols before reaching a definitive conclusion regarding its protective effect. Similarly, the strain and age of the mice can also influence the outcome of experimental study. In addition, DNA vaccination is often more efficient in mice than in large animals and humans which are the target species. So the promising DNA vaccines from various experiments should be tested in animal models that closely mimic the definite host of that disease. But the high animal cost and the limited number of immunological reagents makes trials on target species more difficult. So efforts should also be made to reduce the cost of DNA vaccination to make it commercially viable for use in higher animals and humans. Advancements in vector and antigen design, improved formulations and deliver devices/methods, inclusion of adjuvants and prime boosting strategy have greatly enhanced the immunogenicity of second–generation DNA vaccines. This improved performance has spurred a renewed interest in DNA platform, which is reflected by the numerous ongoing experiments on bacterial DNA vaccines. However, the various optimization strategies of second generation DNA vaccines should be evaluated on the proven antigens of different bacteria to further improve their performance.

CONCLUSION

Many attempts have been made in the last decade to develop a DNA vaccine against many bacterial diseases which are shortly reviewed in the present literature. The results of these vaccination trials are quite encouraging which strongly suggests that DNA immunization can represent an efficient method to fight against many bacterial infections especially against intracellular bacteria in the nearby future itself. But, before becoming a reality, it must still be subjected to further experiments, including the various second generation DNA vaccine optimization strategies.

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