Applied Microbiology and Biotechnology (2020) 104:4273–4280
https://doi.org/10.1007/s00253-020-10548-5

MINI-REVIEW

Use of plasmids for expression of proteins from the genus *Leishmania* in *Escherichia coli*: current state and perspectives

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Received: 13 February 2020 / Revised: 5 March 2020 / Accepted: 15 March 2020 / Published online: 25 March 2020

Abstract

Leishmaniosis is caused by the protozoa of the genus *Leishmania* with a wide spectrum of clinical and epidemiological manifestations which are characterized into four clinical groups: cutaneous, mucocutaneous, diffuse cutaneous, and visceral. American visceral leishmaniosis (AVL) or visceral leishmaniosis (VL) has been known as the most severe form of the disease. However, despite the growing number of people exposed to the infection risk and the great effort done by the scientific community worldwide to significantly increase the knowledge about these diseases, there is no vaccine capable of preventing VL in humans. In this short review, we present some of the plasmids used for the expression of recombinant protein by *Escherichia coli* strains used mainly for the second generation of vaccines for leishmaniosis. It can be emphasized that currently, these vectors and hosts play an important role in developing vaccine strategies against the disease. Indeed, use of the *E. coli* BL21 (DE) strain is remarkable mainly due to its characteristics for being a stable protein producer as well as the use of histidine tags for antigen purification.

Key points

- *Plasmid vectors and E. coli will continue being important for studies about leishmaniosis.*
- *Protein purification exploiting histidine tags is a key technique.*

Keywords *Leishmaniosis* · *E. coli* · Recombinant protein · Antigen · Plasmid

Introduction

Leishmaniosis comprises a complex of diseases caused by the protozoa of the genus *Leishmania* with a wide range of clinical and epidemiological manifestations (Karami et al. 2013; Esteva et al. 2017). Leishmaniosis are typical tropical diseases that, depending on the parasite tropism, may be characterized into four clinical groups: cutaneous, mucocutaneous, diffuse cutaneous, and visceral (Freitas-Junior et al. 2012). American visceral leishmaniosis (AVL) or visceral leishmaniosis (VL) has been known as the most severe form of the disease due to its wide distribution showing high lethality if left untreated for a long time (Jain and Jain 2015; Esteva et al. 2017; Chaukan et al. 2018). Due to expansion of the human population from rural epidemic regions to larger cities, mainly in developing countries, it is estimated that 350 million people are currently at risk of contracting any form of the disease (WHO 2016). It is known that approximately 900,000 to 1.3 million new cases of the disease are estimated to occur annually in 75 countries that could cause approximately 20,000 to 30,000 deaths worldwide (Sousa-Gomes et al. 2017). Dogs (*Canis familiaris*) are considered the main reservoir for AVL, and detection of infected dogs is crucial for controlling human cases of the disease. Although infected dogs may have not clinical manifestation of canine visceral leishmaniosis (CVL), such animals act as a source of intense parasitism for

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phlebotomine sand flies (Michalsky et al. 2007). Strategies for controlling VL are based on early diagnosis, treatment of human cases, canine serology tests, community education programs, and environmental management. However, despite the growing number of people exposed to the infection risks, the high incidence, and the great efforts done by scientific communities worldwide with the intention to significantly increase the knowledge about these diseases, there is still no efficient and safe vaccine capable of preventing VL in humans (Singh et al. 2016; Sousa-Gomes et al. 2017). Treatments performed by the available drugs are toxic and costly (Gillespie et al. 2016). Despite advances in diagnosis and vaccination, and considering that different techniques have been developed for the diagnosis of leishmaniosis, there are no standard tests for AVL antibody detection, i.e., a test that shows 100% of activity of sensitivity for the detection of specific antibodies against *Leishmania*. Identifying the parasite by means of parasitological tests is important since the symptoms could also cross react to other diseases such as malaria, Chagas disease, tuberculosis, among others (Gontijo and Melo 2004).

A safe and efficacious vaccine should elicit long-lasting immunity, limiting the use of medications and finally a potent inducer of a balanced T_H1- and T_H2-mediated immune response, ideal for controlling AVL expansion. For instance, a vaccine promoting protection for 5 years with 90% efficacy would be economically viable when compared with current treatments. As mentioned previously, treatment for AVL relies on anti-leishmania drugs which are costly and toxic with adverse side effects, thus emphasizing the need for safe and effective vaccination strategies. The search for vaccines seems to be the most economical method for AVL prevention (Selvapandiyan et al. 2012).

Leishmania immunodominant antigens have the ability to trigger a specific immune response, and it demonstrates their potential to be included as a vaccine subunit for AVL. The use of new approaches, including immunoinformatics tools for the selection of leishmania immunodominants epitopes, can lead to a specific immune response and seems to be a great strategy for the second-generation vaccines (Ghorbani and Farhoudi 2018) besides the potential of such immunoepitopes for the development of kits with more sensitivity, specificity, and affordability for fields where the disease is endemic (Martins et al. 2006; Gillespie et al. 2016). In this context, the use of protein recombinant expression systems such as vectors and host cells plays an important role in protein folding, since its property can be influenced by several factors in a heterologous system. This mini-review focuses on the use of *Escherichia coli* as host cell and plasmids for the expression of recombinant *Leishmania infantum chagasi* (*L.i.chagasi*) antigens. Additionally, special emphasis is given to *E. coli* strain type and cultivation strategies used during *L.i.chagasi* antigens expression as well as the performed purification strategies. Special attention is also given to plasmid characteristics such as size, promoter, operon control, and tags. Indeed, most studies have been reporting the development of second-generation vaccines against leishmaniosis where the use of recombinant DNA technique and adjuvants makes the final formulation. Additionally, third-generation vaccines which are DNA-based vaccine will also be introduced as the current trend (perspectives).

E. coli as host for *Leishmania* antigen expression

Escherichia coli has been used as host cells for the expression vectors of recombinant proteins for a long time, since this bacterium is well molecular, genetic, and physiologically characterized (Aucoin et al. 2006; Ahmad et al. 2018; Briand et al. 2016; Cheng et al. 2007; Collins et al. 2013; Einsfeldt et al. 2011; Ghorbani et al. 2009; Jhamb and Sahoo 2012; Kaur et al. 2018; Larentis et al. 2014; Marbach and Bettenbrock 2012; Malik et al. 2016; Norsyahida et al. 2009; Rudolph et al. 2010; Tripathi et al. 2009; Vélez et al. 2014; Wang et al. 2010; Wen et al. 2005; Zhang et al. 2009). While searching for recombinant proteins from *Leishmania i. chagasi* as potential vaccine subunits, Martins et al. (2006) expressed the 503 antigen, a protein that shows 100% identity with the elongation factor-1γ from *L. infantum*, identified by double scanning cDNA library of the amastigote form of *Leishmania i. chagasi*. The construction of recombinant proteins used the pQE-30 plasmid and *Escherichia coli* M15 as host.

It should be highlighted that a cellular and humoral immune response was observed after experimental model and serology from endemic AVL areas' individuals. It showed the capacity of the 503 antigen to stimulate the production of interferon gamma (IFN-γ) (Martins et al. 2006), thus the 503 antigen may be a potential immunoantigen to be used in the formulation of kits for specific VL diagnosis. Table 1 shows the main features of the expression system used for the 503 antigen. Our group has explored some other *Leishmania i. chagasi* antigens such as 648 (Vaz et al. 2011, 2015), which shows 97% identity with the A2 antigen (between amino acids 44 and 297), but the studies were conducted intensively for the optimization of 503 antigen expression.

In this context, Vasconcelos et al. (2018) studied the influence of IPTG and lactose as inducer at different concentrations and induction times on the 503 antigen expression by *Escherichia coli* M15 harboring the pQE-30 plasmid and using 2xYT medium (Table 2). It was shown that IPTG at 100.0 μM was able to give a 503 antigen concentration of 0.087 g/L that was 5.6-fold higher than obtained by lactose at 1.0 g/L. Additionally, Ribeiro et al. (2019) studied batch cultures incubated in a shaker also using the 2xYT medium but under different IPTG concentrations (0.01 to 1.5 mM), temperatures (27 to 42 °C), and induction times (0.5 to 6 h

Table 1 Main Expression systems features for *Leishmania* recombinant protein expression using *E. coli*

Recombinant protein	Plasmids/size (bp)	Promoter	Origin	Regulatory element	Selectable marker	Tags and fused proteins	Reference
503 antigen (elongation factor-1γ of *L. infantum*)	pQE30/3400	T5	*E. coli* M15 (derived from K-12)	lacI	Ampicillin	His_6 (N-terminal)	Martins et al. (2006)
A2 antigen	pET 28a(+)/5369	T7	One Shot® Mach1™-T1^R *E. coli* & BL21 (DE), ER2566, *Rosetta*	lacI	Kanamycin (BL21 (DE) and ER2566, Chloramphenicol (Rosetta)	His_6 (N-terminal and C-terminal)	Jusi (2015), Jusi et al. (2015)
K9-K39-K26 chimera	pGex-2T, pGex-6H (modified from pGex-6P)	T7	*E. coli* BL21star	lacI	Ampicillin	His_6 (C-terminal) and GST (N-terminal)	Boarino et al. (2005)
Li-P4 protein	pGEM-T easy vector/ 3015, pET 28a/5369	T7	*E. coli* DH5α, BL21	lacI	Ampicillin	His_6 (C-terminal)	Farajnia et al. (2011)
NH36 nanovaccine	pVAX1-NH36/3936	CMV	*E. coli* DH5α	lacZ	Kanamycin	Untagged	Ureña-Búrquez et al. (2019)
P36 protein	pRSET-P36/1000	T7	*E. coli* TOP10 & BL21-Gold (DE)	lacZ	Ampicillin	His_6 (N-terminal)	Hammoudeh et al. (2015)
PDI-2 protein	pET-15b/5708	T7	*E. coli* DH10B & BL21 (DE)	lacI	Ampicillin	His_6	Ali et al. (2016)
rLc36 protein	pGEM-T easy vector /3015, pET 28a(+)/5369	T7	*E. coli* DH5α, BL21 (DE)	lacI	Kanamycin	His_6	Nogueira (2014), Nogueira et al. (2018)

Table 2 Cultivation and purification strategies for recombinant proteins of *Leishmania* using *E. coli*

E. coli strain /recombinant protein	Medium cultivation	Inducer/concentration (mM)	Induction strategy	Metal/IMAC	Protein concentration (mg/L)/molar mass (kDa)	References
E. coli BL21/Li-P4	Luria-Bertani (LB)	IPTG/1.0 mM	Induction at optical density at 0.5	Nickel/nitrilotriacetic acid (NTA)	Not shown/33	Farajnia et al. (2011)
E. coli BL21star/ K9-K39-K26 chimera	Not shown	IPTG/0.5 mM	Early log phase for 2 h with 1.0 mM IPTG	Glutathione S sepharose 4B, and nickel Hi Trap chelating HP column	10/45	Boarino et al. (2005)
E. coli BL21 (DE)	Luria-Bertani (LB) 1.0% tryptone, 0.5% yeast, 1.0% NaCl)	IPTG/0.5 mM	Induction at optical density (0.5–0.7) cultivation for 16 h after induction at 18 °C	Nickel/not shown	600/51	Ali et al. (2016)
E. coli BL21 (DE), ER2566, *Rosetta*/A2	Luria-Bertani (LB) containing 0.2% glucose	IPTG/0.5 mM	Induction at optical density (not shown) cultivation for 6 h after induction (ER2566, *Rosetta*) and 3 h for BL21 (DE) at 37 °C	Not shown	334 (BL21 (DE)), 328.25 (ER2566), 411.5 (*Rosetta*)/11	Jusi et al. (2015)
E. coli BL21 (DE)/rLc36	Luria-Bertani (LB)	IPTG/0.5 mM	Induction at optical density (0.5) cultivation for 4 h at 30 °C	Nickel/chelating sepharose TM Fast Flow	Not shown/28.4	Nogueira (2014)
E. coli BL21-Gold (DE)/ P36 protein	Luria broth (1.0% tryptone, 0.5% yeast, 1.0% NaCl)	IPTG/0.5 mM	Induction at optical density (0.5–0.7) cultivation for 16 h after induction at 37 °C	Nickel/nitrilotriacetic acid (NTA) superflow sepharose	500/36	Hammoudeh et al. (2015)
E. coli DH5α/pVAX1-NH36*	Terrific broth (glycerol 13 g/L, vitamins, yeast extract 24 g/L, tryptone 12 g/L, KH_2PO_4 2.31 g/L, K_2HPO_4 12.31 g/L	Not shown	For plasmid production optical density (9.0) cultivation for 9 h after at 37 °C	Not assayed	Not assayed	Ureña-Búrquez et al. (2019)
M15/503 antigen (elongation factor-1γ of *L. infantum*)	2xYT (tryptone 12.0 g/L, yeast extract 10.0 g/L, NaCl 5.0 g/L)	IPTG/(0.01–1.0)	Induction at instant (0.5-6 h) at temperature (27–42 °C)	Nickel/streamline chelating	(18–119)/56	Ribeiro (2018), Ribeiro et al. (2019)
M15/503 antigen (elongation factor-1γ of *L. infantum*)	2xYT (tryptone 12.0 g/L, yeast extract 10.0 g/L, NaCl 5.0 g/L)	IPTG/(0.02–1.5) and lactose (0.29–29.2)	Induction at optical density (0.5–0.7) cultivation for 16 h after induction at 18 °C	Nickel/streamline chelating	(28–87)/56	Vasconcelos et al. (2018)

*Plasmid harboring the NH36 gene to be applied to DNA vaccine

after the start of the culture), as shown in Table 2. It was shown that IPTG toxicity caused a metabolic stress in the cells as well as instability (Silva et al. 2012; Tomazetto et al. 2007) of the plasmid pQE-30, due to the metabolic burden imposed by the expression of the recombinant protein (Lecina et al. 2013; Ribeiro 2018). In this case, optimal conditions for the 503 antigen expression from *Leishmania i. chagasi* in *Escherichia coli* M15 were at a temperature of 37 °C with induction time of 2 h using an IPTG concentration of 1.0 mM, leading to a maximum antigen concentration of 0.119 ± 0.009 g/L.

Regarding purification strategies, the most concerning factor that interfered in 503 protein purification was difficulty in lipopolysaccharide (LPS) removal from the *E. coli* M15 strain. However, Sousa Junior et al. (2015, 2017) and Leitão et al. (2018) showed that it is possible to use Triton X-114 surfactant to remove LPS at the washing step during an expanded bed adsorption (EBA) protocol with LPS removal with great efficacy (> 99.0%). Leitão et al. (2018) also showed that even though nickel is the most used metal in immobilized metal affinity chromatography (IMAC) purification protocols for the 503 antigen, Cu^{+2} was the metal with the highest adsorption capacity.

Strategies have also been developed for the detection of infected dogs once CVL imposes a negative impact on population health. In order to develop for a more sensitive and specific recombinant protein-based immunoassay with high capacity for detecting asymptomatic carriers, parasite-specific recombinant antigens such as rK39, rK26, and rA2 have been tested (Porrozi et al. 2007; Arruda et al. 2016). In this context, Jusi et al. (2015) also reported the expression of recombinant A2 family genes of *Leishmania chagasi* (Jaboticabal strain) that could be useful as a tool for CVL diagnosis using three *E. coli* BL 21 (DE), ER2566, and *Rosetta* strains (Table 1). The expression vector pET 28a containing A2, the target leishmania antigen sequence, was transformed into *E. coli* BL 21 (DE), ER2566, and *Rosetta*. As commented by Jusi et al. (2015), gene expression of the A2 family mainly occurs at the amastigote stage but not during the promastigote one. Additionally, there is evidence that this antigen is produced in response to stress, thus being important for the survival of *Leishmania donavani* in visceral organs.

In relation to rK39 (Lima et al. 2017), Boarino et al. (2005) produced a recombinant chimera consisting of a fusion of K9, K39sub, and K26 antigens which was expressed in *E. coli* BL21star. In this case, plasmids pGex-2T and pGex-6H (modified by the authors based on the pGex-6P) contained a six-histidine tag (Table 1). Once both plasmids could express the gene of interest in fusion with glutathione-S-transferase (GST) under the control of the inducible *tac* promoter, the fusion protein could be purified by affinity chromatography using glutathione sepharose and by IMAC as well, thus the chimera could be purified by double affinity steps (Table 2).

Alongside screening for leishmania antigens as potential vaccine candidate against AVL, efforts have also been looking for antigen candidates against cutaneous leishmaniosis (CL). In this context, Hammoudeh et al. (2015) reported the expression of the antigen P36 (Lack), a protein expressed on the surface of *Leishmania tropica* amastigotes forms. This immunogenic antigen (36 kDa) is the leishmania form that can survive inside of mammals' macrophages (Ramos et al. 2008, 2009). P36 (Lack) was expressed in *E. coli* BL21-Gold (DE) harboring the pRSET-P36 plasmid as shown in Table 1. Purification of the P36 (Lack) protein was performed by His_6-tag using nickel and the nitrilotriacetic acid (NTA) superflow sepharose. Farajnia et al. (2011) searched for a molecule that could be used as target for chemotherapy to treat VL; they expressed a Li-P4 nuclease belonging to class I of nucleases in pET28a and transformed into *E. coli* BL21 (Table 1). The expressed recombinant P4 molecule (33 kDa) was also purified by His_6-tag using nickel and the nitrilotriacetic acid (NTA) column, demonstrating that this strategy is efficient for protein expression and purification (Table 2). Additionally Nogueira (2014) and Nogueira et al. (2018) expressed an antigen rLc36 of unknown function containing a nucleotidyl transferase important for protecting *Leishmania* amastigotes against reactive oxygen species produced by macrophages (Nogueira et al. 2018). According to Nogueira (2014), it was possible to express rLc36 using the vector pET 28a(+) and the strain *E. coli* BL21 (DE) (Table 1) in order to obtain a protein with 28.4 kDa (Table 2).

DNA-based vaccines using expression plasmids (third-generation vaccines) have been explored in the field of immunization against infectious diseases and are safe for both humans and animals. Besides that, DNA vaccines have a rapid and inexpensive production, shelf stability, safety use, storage, and transportation. The system also has advantages, including their ability to stimulate the innate and adaptive immune responses.

New perspectives and current applications in the field of vaccinology against leishmaniosis are due to the use of such plasmids for protein expression from the genus *Leishmania* using *Escherichia coli* for the development of nanovaccines based on DNA vaccine systems (Ureña-Búrquez et al. 2019). This approach, i.e., plasmids as vectors and *E. coli* as host cells, plays a key role in the third-generation vaccines. As shown in Table 1, Ureña-Búrquez et al. (2019) used PEGylated liposomes as nanoparticles (NP) loaded with the plasmid pVAX1-NH36, propagated in *E. coli* DH5α, to be used as a vaccine against leishmaniosis. NH36 is an enzyme vital to *Leishmania* releasing nitrogenous base from foreign DNA used in the synthesis of parasite DNA. For this reason, this recombinant enzyme may be a good candidate for a Plasmidial DNA (pDNA) vaccine since it has been tested in experimental models of the disease (Ureña-Búrquez et al. 2019). In addition, conditions used to express this target gene are shown in Table 2.

Conclusion

Leishmaniosis represents one of the major neglected diseases in the world. ALV has undergone an extensive process of urbanization in the outskirts of medium and large cities. Most individuals infected with the leishmania develop a protective Th1-lymphocyte-mediated immune response against the pathogen, and still, there is not yet a safe and effective vaccine to control leishmaniosis in humans. Therefore, scientists are not measuring efforts in order to develop a vaccine that contains specific pathogen antigens for vaccine subunits, such as the second generation one, i.e., in which a poly-epitope-based DNA immunization approach can lead to an excellent ability to induce T cell responses against *Leishmania*. In this context, plasmid vectors as well as *E. coli* strains play a key role for this approach. During protein expression, different strategies are used. However, it has been observed that the use of *E. coli* BL21 (DE) is remarkable, mainly for its characteristics of being a stable protein producer. Additionally, protein purification by using histidine tags is present in the majority of the approaches reviewed in these studies. The development of nanovaccines based on pDNA still relies on these two important elements, the vector and the host cells, thus it is expected that they will assist researchers for a long time in the development of new genetic engineering in the field of vaccinology.

Acknowledgments The authors acknowledge Dr. Mary Wilson (Iowa University/USA) for providing the microorganism strains at Chemical Engineering Department/UFRN.

Author contribution VTR, ALOSL, LTCPV, MAOF, FCSJ, and ESS conceived and contributed to the literature search. FCSJ, DRAM, and ESS wrote the manuscript. All authors read and approved the manuscript.

Funding information The authors thank Coordination for the Improvement of Higher Education Personnel (CAPES) and Brazilian National Council of Research (CNPq) for the financial support (Grant 401817/2016-4 and 305251/2017-1).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants This article does not contain any studies with human participants or animals performed by any of the authors.

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