

Investigation *ctpA* Gene Among *Listeria Monocytogenes* and Transferring it into *E.coli*

Jamileh Nowroozi, Ph.D. ☆ ‡

☆ Microbiology Department, Iran Medical Sciences University

‡ P.O.Box: 14155-6183, Microbiology Department, Iran Medical Sciences University, Tehran, Iran

Abstract

Introduction: *Listeria monocytogenes* is a Gram positive bacterium, often found in the environment and is responsible for serious food-borne diseases such as perinatal infections, septicaemia and meningoencephalitis in humans and animals. For this reason, distribution of the *ctpA* (copper transport protein A) among *L. monocytogenes* was isolated from clinical, environment, dairy, and poultry samples, and was investigated. Then, *ctpA* gene was transferred into *E.coli* DH5- α .

Materials and Methods: This investigation was carried out in two steps (*ctpA* was found in *L. monocytogenes* isolated from different sources, which was kept in culture collection of Adelaide University, Australia. Then *ctpA* gene was transferred into *E. coli* DH5- α . *CtpA* DNA from *L. monocytogenes* was amplified by PCR, identified on agarose gel, purified by phenol, and ligated into pGEM-T vector. Then, it was transferred on X-gal plate containing ampicillin. The sequencing of *ctpA* DNA was determined by using dye terminator kit to purify DNA, and followed by sequencing machine.

Results: By using PCR to identify the homologous DNA in 69 isolates, 38% of tested isolates contained *ctpA* determinant. Our results showed that: 90% of clinical and dairy isolates; 85% of environmental isolates and 7% of poultry isolates of *L. monocytogenes* contained *ctpA* in chromosome DNA. Fortunately, the transformation of *ctpA* from *L. monocytogenes* into *E. coli* DH5- α was successful.

Conclusion: Since, the existence of *ctpA* in clinical, dairy and environmental samples was 90% and in poultry was 7%, therefore, the virulence of all strains of this bacteria are not the same. Introducing such clone (*ctpA* gene) into suitable carrier strains, could be expected to produce a good oral immunogen against *L. monocytogenes*.

Key words: *Listeria monocytogenes*, *E. coli* DH5- α , transformation gene, *ctpA* gene.



Introduction

The Gram-positive *Listeria monocytogenes*, is a facultative, non-spore forming, rod shaped and intracellular organism. This bacterium is found in the environment. Epidemiological studies of listeriosis outbreaks have suggested butter (1), soft cheeses (2), pasteurized milk (3), raw vegetables (4), meat and production of meat (5) as probable vehicle of infections in human. Elderly patients, pregnant women and individuals with predisposing conditions that compromise their immunity, such as transplants, and malignancies such as lymphomas, are at a particular risk. Meningitis is the most commonly recognised form of human listerial infection and it demonstrates the tropism of *L. monocytogenes* for the central nervous system (6). In perinatal listeriosis, depending on the time of neonatal infection, two distinct forms of clinical syndromes are observed. The first is infection of the fetus which often leads to abortion or stillbirth. The second syndrome (late onset neonatal infection), which results from a later infection at or shortly after birth to third week of life, which manifests into meningitis (7).

Francis and Thomas (8) identified a gene in *L. monocytogenes* (*ctpA*), which encodes a 653 amino acid polypeptide with strong similarity to cation transporting p-type ATPases (Genbank Accession Number: U15554). Sequence and phylogenetic analysis, indicated that CtpA was most closely related to a class of p-type ATPase involved in copper transport in procarotytes and eucaryotes. Of *L. monocytogenes* infections (9, 10), which was considered as virulence factors known to play a role in pathogenicity. However, additional factors which affect cell viability may also play a role in allowing the organism to selectively establish an infection. For example, a significant number of *L. monocytogenes* strains are resistant to heavy metal cations including cadmium (11). In this case, resistance is conferred by the *cadA* determinant, a p-type ATPase protein involved in cadmium transport.

P-type ATPases involved in the transport of cadmium or copper ions in other bacteria was reported including *Staphylococcus aureus* *cadA*, *cadC* (12), *Synechococcus* *pacS* (13), *E. hirae* *copA* (14), *H.*

pylori *copA* and ORF (15). These ATPases showed significant similarity to the proteins associated with copper metabolism disorders in humans, Menkes disease (16) and Wilson disease (17). In Menkes disease, the export of copper from intestinal cells is defective, and results in severe copper deficiency. In contrast, Wilson disease results from a failure in removing copper from the liver into bile, leading to copper toxicity. Interestingly, the CtpA ATPase from *L. monocytogenes* has significant identity to bacterial proteins involved in translocation of copper, as well as those encoding Menkes and Wilson diseasesdeterminants (10). Given *L. monocytogenes* is ubiquitously distributed throughout the environment, which suggests an important role for the CtpA p-type ATPase in survival of this organism in its natural habitat, and may involve maintaining copper homeostasis through the transport of copper ions. The aim of this study was concerned with the analysis and distribution of a copper transport gene (*ctpA*) from different sources (clinical, dairy, environment and poultry) obtained from culture collection at Adelaide University, Australia, and then to transfer this gene into *E. coli* DH5- α .

208

Materials and Methods

This investigation was carried out in 2 steps. first, *ctpA* was found in *L. monocytogenes* isolated from different sources at culture collection laboratory in the Microbiology Department of Adelaide University, Australia and then the *ctpA* gene was transferred into *E. coli* DH5- α (8, 18).

All these bacteria were kept in solution of glycerol and peptone solution at -70°C. Fresh single colonies of *Listeria* were prepared by streaking a loopful of glycerol stock onto Brain Heart Infusion agar and were incubated overnight at 37°C. For routine use, cultures were maintained on agar plate at 4°C.

First, chromosomal DNA from *Listeria* spp. was extracted by modification of Flamm et al. method (19). DNA concentration was determined by measuring the OD of the DNA solution at 260 nm using a spectrophotometer. Electrophoresis was performed at room temperature on horizontal 1% (w/v) agarose gel



in a TAE buffer solution (Tris, Acetic acid and EDTA). The gel was followed by staining in ethidium bromide. DNA fragments were visualised by transillumination using a UV Transilluminator and photographed on polaroid 667 positive film.

The size of fragments obtained by restriction enzyme, was calculated by comparing their relative mobility to EcoR1 digested *B. subtilis* bacteriophage SPP1 DNA. *L. monocytogenes*. Preliminary experiments were done to determine optimum PCR modification condition. Then, *ctpA* in 69 samples was investigated, it was obtained from culture collection laboratory in Microbiology Department of different sources (environment, patients, dairy, meat and poultry). Plasmid pGEM-7 Z F (+) was used as vector to clone DNA fragments of *L. monocytogenes* (20). *Escherichia coli* DH5- α was used as host strain for recombinant clones, because *ctpA* was not detected in this bacteria. In this study, *ctpA* DNA from *Listeria* was amplified by PCR (8), then, ran on agarose gel electrophoresis and purified with phenol. Electrophoresis fragment DNA was cut without using ethidium bromide or UV. One ml phenol/gram agarose gel was vortexed and kept at 70°C for 1hr. After centrifugation, chloroform was added and DNA was obtained by 100% ethanol. Fragments containing portions of the *ctpA* genes were ligated into pGEM-T vectors, essentially by published procedures. Plasmids containing inserts were introduced into *E. coli* DH5- α by pretreating cells with CaCl₂ to render their competent cells. Insert containing plasmids were identified on nutrient agar plates containing galactosidase substrate and chromophore x-gal 40 g/ml. White colonies were restreaked on ampicillin plate and one colony was picked up, then DNA chromosome was extracted, and amplified with PCR. Dye-terminator kit was used to purify *ctpA* DNA. Then, the DNA sequencing was performed by automatic sequencer (21).

Results

Preliminary experiments indicated that the optimum conditions were using 25mM MgCl₂ and the following PCR temperature cycling: 94°C (templated

denaturation) for 3 min (1cycle), 55°C (annealing) for 30 seconds, 72°C (extension) for 3 min (30 cycles), 72°C (stabilization) for 4 min (1cycle) and then holding at 4°C (1cycle) (22). Of 69 *L. monocytogenes* isolates, in 38% cases, 558 bp amplification products was detected. The results showed that 90% of clinical and dairy isolates, 85% of environmental isolates and 7% of poultry isolates of *L. monocytogenes* contained *ctpA* in chromosome DNA. PCR analysis indicated, that *ctpA* homologous DNA was restricted to some strains of *L. monocytogenes*.

Hpa-1 digestion of amplified DNA fragment produced two products: 176 bp and 382bp (0.558 kbp), when compared with DNA of SPP1 on agarose gel electrophoresis. After ligated chromosomal DNA fragment into pGEM-T and transforming into *E. coli* DH5- α competent cells, at least 200 white transformed colonies initially screened by blue/white differentiation in x-gal plates appeared. Positive clones (white colonies) were identified during growth on media containing x-gal by their ability to produce galactosidase. White colonies contained plasmid DNA were picked up and cultured on nutrient agar plates which contained ampicillin. Then, DNA from the transferred bacteria was isolated, amplified by PCR and purified by agarose gel. By using dye-terminator kit, *ctpA* was purified and sequencing was performed by automatic sequencer. our results indicated that the complete nucleotide sequences of *ctpA* in transferred *E. coli* was exactly the same as the *ctpA* in *L. monocytogenes*. Therefore the transformation was successful. In Fig 1, *ctpA* band detected on agarose gel electrophoresis is shown.

Discussion

Copper is an essential nutritional requirement for living cells, yet it is very toxic when present in excess. It is likely that the bacteria have essential copper transport systems responsible for maintaining copper ions at a relatively constant low level (23). Proteins involved in transporting copper or cadmium in some other bacteria was reported (11, 15). During infection, eukaryotic host cells are exposed to dramatic changes in trace element concentration in the serum (24). For



example copper ion concentration in rat liver significantly decreased during parasitic infection (25, 26). CtpA may therefore, play a critical role in the selective adaptation of *L. monocytogenes* to varying environments including the intracellular location of an infected host. For survival in this environment, *L. monocytogenes* may have acquired the mechanism to sequester copper from infected cells by ctpA function.

CtpA is necessary for *L. monocytogenes* to persist in organ tissue of infected mice (18). In view of the significance of ctpA in bacterial infections, we investigated the distribution of the ctpA among *L. monocytogenes* isolates from environment, clinical, dairy and poultry samples, which was kept in culture collection laboratory in Microbiology Department of Adelaide University, Australia. Using PCR to identify homologous DNA in 69 isolates, 38% of the tested isolates contained the ctpA determinant. However, DNA homologous to ctpA was not detected in all strains.

Antibiotics have effectively been used to treat human listeriosis (27). Ampicillin, amoxicillin and gentamicin were considered the primary choice for treatment of human listeriosis, but antibiotics such as chloramphenicol and azlocillin had no effect. Nevertheless, it is acknowledged that up to 30% of the patients who have previously had drug therapy, will still

succumb to listeria infection (28). It is likely that the intracellular habit of *L. monocytogenes* protects the organisms from exposure to antibiotics. Consequently, a number of researchers sought to develop a *L. monocytogenes* vaccine that provides a high degree of protection with minimal or no side effects, against infection. The intracellular habit is effectively utilized by *L. monocytogenes* against antibody mediated defense mechanisms. Therefore, a cell mediated response is required for antimicrobial protection. Protective immunity is best created by live vaccines, whereas soluble proteins, and killed vaccines are generally not sufficient (29). Presently, only two live vaccines active against intracellular pathogens are in use: *Mycobacterium bovis*, BCG (Bacillus Calmette- Guerin) against tuberculosis and *Salmonella typhi* Ty2la providing protection from typhoid fever (30).

In this investigation, transformation of ctpA from *L. monocytogenes* into *E. coli* was successful. After expression of ctpA in *E. coli* (produced protein), the antibody was raised against *E. coli* that harbor this clone, then, the mouse model system protection will be determine. Introduction of such clone into suitable carrier strains could be expected to produce good oral immunogen against *L. monocytogenes*.

210

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211

