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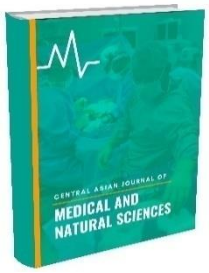

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Evaluation of Internalin-C Role in *L. Monocytogenes* Infection and its Correlation with Exocyst Receptor in Diarrhea Patients using Rabbit Intestinal Loop Model

1. **Hussam Abdul Zahra**
2. **Nawar Al-Janabi**
3. **Naktel Faaz**

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[1,2,3] Department of Biotechnology, College of Biotechnology, Al-Qasim Green University, Babylon, Iraq

Abstract: Listeria monocytogenes is an intracellular bacterium causes many diseases, such as septicemia, brain infection, abortion, and perinatal infection. Internalin-C (InlC) is a virulence gene present only in pathogenic Listeria and InlC mutant Listeria is significantly less virulent. InlC is responsible for facilitating the spread of listeria across host cells. In eukaryotic cells, intracellular vesicles are trafficked to specific sites in the plasma membrane through a multicomponent complex called 'exocyst' where the InlC of L. monocytogenes co-opt to promote internalization of the bacterium within the cells and tissues. However, the aim of this study was to evaluate the role of InlC in L. monocytogenes infection and its correlation with exocyst receptor in diarrhea patients using the rabbit intestinal loop model. To achieve our aim, two intestinal loops were constructed surgically in a live rabbit, and the first intestinal loop was injected by 1 ml of 107 CFU/ml of L. monocytogenes, and the second intestinal loop was injected by 1 ml of (PBS) as a control. Result showed that expression levels of InlC was significantly high in L. monocytogenes injected into the intestinal loop (fold- 5.3499) compared to expression levels of InlC in L. monocytogenes grown on (BHI) agar as a control (fold- 1.0143), (p-value 0.001). Also, the expression levels of exocyst receptor was highly significant in the rabbit intestinal tissues injected by L. monocytogenes (fold- 2.3436) compared to expression levels of exocyst in the intestinal tissues injected by (PBS) as a control (fold- 1.0461), (p-value 0.017). The present study offers a useful method for comprehending the relationship between the host and pathogen as well as the pathogenicity of L. monocytogenes during infection.

Key words: Listeria monocytogenes, (InlC), exocyst, RT-qPCR.

INTRODUCTION

Listeria monocytogenes is a Gram positive food born pathogen and responsible for the one of the worst form of food poisoning, listeriosis that may affect both people and animals. Out of 21 species presently known in the genus *Listeria*, only *L. ivanovii* and *L. monocytogenes* are considered to be mammalian pathogens (1). Once inside the host, *L. monocytogenes* utilizes a wide variety of complex pathways for invasion of eukaryotic cells, intracellular survival, immunological evasion, and systemic dissemination (2, 3). Additionally, this bacterium may pass the blood-brain and placental barriers, leading to dramatic disease development (meningitis, abortion) and a fatal end in immunocompromised persons and pregnant women respectively (4) . *Listeria* infection in humans may range from mild to fatal depending on the aggressiveness of the bacteria, the quantity of bacteria consumed, the genetic variety of a population, the host's overall health and the host immune system (5). Virulence factors of *L. monocytogenes* are either scattered across the genome for example, InlA, InlB, InlC, lapB, or clustered in the pathogenicity islands like LIPI-1, LIPI-3, and LIPI-4. *L. monocytogenes* has 24 internal protein families, each identified through the presence of leucine-rich tandemly ordered repeats (LRRs) at the amino termini (6). The process of adherence and entry of *L. monocytogenes* to the host cells are primarily controlled by two subfamilies of these internal proteins (7). The first sub-family is the large external proteins (70–80 kDa), which include the internalin-A (InlA) and internalin-B (InlB) (8). The other sub-family is the lesser external proteins (25 to 30 kDa), which include the internalin-C (InlC), internalin–D (InlD), internalin–E (InlE), internalin-F (InlF), internalin-G (InlG) and internalin-H (InlH) (7).

However, the dissolved internalin-C protein (InlC) is responsible for stimulating the production of eukaryotic membrane protrusions, which is responsible for facilitating the spread of *L. monocytogenes* across host cells. It was reported that when *L. monocytogenes* successfully evade mammalian cell phagosomes, the expression of InlC is highly induced (9). The soluble InlC protein promotes the formation of eukaryotic plasma membrane protrusions by interacting with the cell scaffolding protein Tuba and the exocyst complex (10). In addition to stimulate the protrusion formation and spread, InlC also associates with host IKK-α to attenuate the innate immune response (11). InlC, also called IrpA in both *L. monocytogenes* and *L. ivanovii*, which is a novel set of leucine- rich repeat (LRR) protein that has been discovered (12).The C-terminal repeat region, the LPXTG motif and the membrane anchor seen in the big internalin are absent in InlC. Therefore, the internalin proteins of this family are typically smaller (about 30 kDa against the 80 and 71 kDa of InlA and InlB, respectively) and discharged in soluble form into the culture supernatant (13). Therefore, InlC is the just secreted internalin found in *L. monocytogenes* to date and mostly expressed in the cytoplasm, particularly during the later stages of an infection, when bacteria are engaged in the process of actively spreading across cells in the host (14).

Further, exocysts are multicomponent complexes that are found in eukaryotic cells. They are responsible for the transport of intracellular vesicles to certain locations in the plasma membrane (15,16). Eight proteins make up the exocysts, which include Exo70, Exo84, Sec3, Sec5, Sec6, Sec8, Sec10 and Sec15 (17). Almost every known living thing, from fungi to mammals to plants to even certain protozoa, has this complex (18, 19). The exocyst attaches vesicles from the trans-Golgi network (TGN) or the recycling endosome (RE), two endosomal compartments, to the plasma membrane (16). Recent findings showed that InlC influences host polarized exocytosis as well as cortical tension to facilitate the propagation of *L. monocytogenes* in Caco-2 BBE1 cells (10). Using an exocytic probe, *L. monocytogenes* induces exocytosis in protrusions in a way that is dependent on InlC. Also, InlC is required for the recruitment of Exo70 to bacterial protrusions and forms an association with the exocyst protein Exo70 in co-precipitation assays. It is still unknown if InlC interacts directly with Exo70 or when it joins a complex with this exocyst protein via a middleman host protein. Significantly, RNAi-mediated Exo70 or other exocyst component reduction affects *L.*

monocytogenes cell-to-cell dissemination as well as the frequency and height of bacterial protrusions (10). It is notable that two separate internalin protein molecules, InlB and InlC take advantage of the exocyst and promote various phases of *L. monocytogenes'* intracellular life cycle. Exocysts encourage the expansion of plasma membranes in a variety of activities, such as cell migration, neurite branching, phagocytosis, and ciliogenesis (17). However, the aim of this study was to understand the role of InlC in *L. monocytogenes* infection and its correlation with exocyst receptor using the rabbit intestinal loop model.

Materials and methods

Bacterial strain and animal experiments

Listeria monocytogenes used in the study were isolated from stool samples of patients with diarrhea at Al-Qasim Green University (20). *L. monocytogenes* was grown on brain heart infusion (BHI) agar and broth at 30 ^{0}C to the optical density of 600 nm (OD_{600}) and suspended in 1X phosphate-buffered saline (PBS) to 10^{7} CFU/ml at room temperature. Animal experiments were conducted under a guideline approved by Al-Qasim Green University and as a previously described procedure (21). Briefly, 5 specific pathogen free rabbits (3 years old with mean weight 2.5 kg ± 10 g) were obtained from the hatchery of the Department of Medical Biotechnology at Al-Qasim Green University and used in this study. The rabbits were anesthetized with 10% ketamine (1.5ml/kg) and xylazine (1ml/kg) and their small intestines were isolated. Two intestinal loops of about 3-4 cm each were constructed by double ligation in each rabbit. The first intestinal loop was injected by 1 ml of a 10^{7} CFU/ml dosage of *L. monocytogenes* and the second intestinal loop was injected by 1 ml of (PBS) as a control. After the injection, the rabbit's intestines were returned back and the rabbit's walls were sutured closed. The rabbits were then remained alive for 8 hours to allow the intestines to respond to *L. monocytogenes*. Rabbits were then sacrificed by intramuscular injection of 10 ml/kg of chloroform. From sacrificed rabbits, the intestinal loops were isolated and *L. monocytogenes* was collected from the intestinal loops and directly placed in tubes containing RNA later. Also, the loops tissue were washed with cold PBS and directly placed in tubes containing liquid nitrogen. All the experiments included in this study were performed in 5 replicates.

Total RNA extraction and cDNA synthesis

The RNA extraction kit (GENEzolTM TriRNA Pure kit (Geneaid) was used to extract the total RNA from collected *L. monocytogenes* to determine the expression levels of InlC. Also, Total RNA from *L. monocytogenes* grown on Brain Heart Infusion broth (BHI) was extracted and used as a control. Further, RNA from loops (tissues) injected by *L. monocytogenes* was extracted to determine the expression levels of exocyst and also from loops (tissues) injected with (PBS) and used as a control. Nanodrop (NanolytiK, Germany) spectrophotometer was used to determine the final RNA concentration, and agarose gel electrophoresis was used to determine the purity of extracted RNAs. The RNAs were then transcribed to cDNA using an (AccuPowerR RocketScriptTM RT PreMix) kit as directed by the manufacturers.

Quantitative real-time PCR and data analysis

The relative expression levels of Internalin C (InlC) and its receptor exocyst were determined using Green starTM kit (Bioneer) on a Q3200 (Bio-Gener). The primers set were designed using Primer 3 software and made commercially (Scientific Researcher CO) Table 1. RT-qPCR reaction involved of (20 µl) of total volume containing (5 µl) of cDNA, (11 µl) of DDW, primers (1 µl each) and (2 µl) of SYBR Green MIX. The thermal cycling setup for all genes was 1 cycle at 95°C for 1 min , 40 cycles at 95°C for 5 sec. and at 55°C for 40 sec. and melting 1 cycle at 95°C for 30 sec. and 60°C for 30 sec. The relative expression level of InlC and exocyst was calculated using comparative ΔCt method (Livak method $2^{-\Delta\Delta CT}$). 16s rRNA housekeeping gene was used to normalize the relative expression

levels of InlC. Also, 18s rRNA housekeeping gene was used to normalize the relative expression levels of exocyst.

Table (1): Gene ID and primers used for RT-qPCR in this study

Gene	The sequence of primer(5'- 3')		Gene ID	Amplicon length
InlC	F	ATGTAGATGGTTGTGTCCTGTG	985945	106
	R	CCATCAAATATAGCCTCAGTCTCC		
exocyst	F	CTTCTCTGTGTCTCTGCCTTTAG	100348845	100
	R	GGAGTGAACCAACGGATAGAAG		
16s rRNA	F	GGTGGAGCATGTGGTTTAATTC	X56153.1	320
	R	TTCGCGACCCTTTGTACTATC		
18s rRNA	F	CTGAGAAACGGCTACCACATC	NR-033238.1	107
	R	GCCTCGAAAGAGTCCTGTATTG		

Statistical analysis

Statistical analysis was performed using SPSS software. The two tailed (T- test) was used to assess the differences between the expression levels of InlC in *L. monocytogenes* injected into the rabbits intestinal loops compared to *L. monocytogenes* grown on (BHI) agar. Also, the T- test was used to assess the differences between the expression levels of exocyst in intestinal loops tissue injected by *L. monocytogenes* compared to the intestinal loops tissue injected by PBS. $P < 0.05$ was considered statistically significant.

Results

The RT-qPCR method was applied to assess the expression levels of InlC and its receptor exocyst. Result indicated that expression levels of InlC was significantly high in *L. monocytogenes* injected into the rabbits intestinal loops (fold- 5.3499) compared to *L. monocytogenes* grown on (BHI) agar (fold- 1.0143), (p-value 0.001). Also, the expression levels of exocyst in the rabbits intestinal tissues injected by *L. monocytogenes* was significantly high (fold- 2.3436) compared to expression levels of exocyst in the rabbits intestinal tissues injected by (PBS) (fold- 1.0461), (p-value 0.017). Table (2), Figure (1).

Table (3.1): Gene expression changes of (InlC) and exocyst

Target gene	Control fold change	Infected fold change	T.test	P.value
InlC	0.712025	5.35171	12.293	< 0.001
	1.028114	5.938094		
	1.148698	4.027822		
	1.132884	5.735821		
	1.049717	5.696201		
Mean± SE	1.0143±0.07904	5.3499±0.34371		
SD	0.17673	0.76856		
exocyst	1.219255	3.747686	2.986	0.017
	1.608817	2.43851		
	0.874179	2.421666		
	0.787854	1.554015		
	0.740207	1.554018		
Mean± SE	1.0461±0.16378	2.3436±0.40249		
SD	0.36621	0.89999		

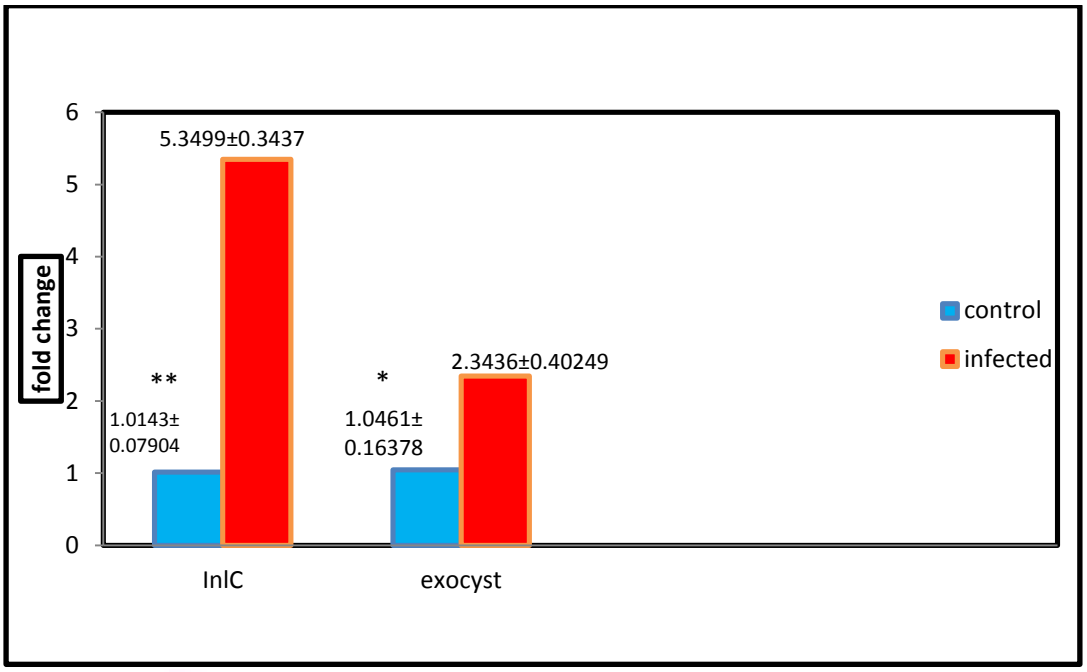

Figure (3.1): Gene Expression changes of InlC and exocyst

Discussion

This study aimed to evaluate the role of InlC in *L. monocytogenes* infection and its correlation with exocyst receptor in diarrhea patients using the rabbit intestinal loop model. The rabbits intestinal ligated loop model is often used in research to characterize human diseases and infections. The RT-qPCR method was applied to assess the expression levels of InlC in *L. monocytogenes* and the expression levels of exocyst in intestinal loop tissues after 8 hours post-infection by *L. monocytogenes*. Our result showed that both InlC and its receptor exocyst are significantly expressed indicating that *L. monocytogenes* expresses its InlC protein during the infection and this protein binds to its specific receptor exocyst on the surface of intestinal cells, which ultimately leads to facilitate the bacterial entry into the intestinal epithelium and propagate within the host cells. This hypothesis is previously proved by a study that showed the important roles of internalin proteins InlA, InlB, and InlC in inducing entry, or intercellular propagation via exocytic routes (22). However, our study showed that InlC has a crucial role in promoting bacterial invasion into intestinal host cells as it was significantly expressed. The fact that InlC is effective in promoting invasion into intestinal epithelial cells is noteworthy, as *L. monocytogenes* infection often targets the intestine as a primary site of infection. This aligns with previous research highlights the importance of InlC in intestinal infections caused by *L. monocytogenes* (23). However, our findings demonstrated a significant increase in InlC expression levels in *L. monocytogenes* when injected into the rabbit intestinal loops (fold- 5.3499), compared to its expression levels in *L. monocytogenes* cultured on BHI agar as a control (fold- 1.0143) (p-value 0.001). This result highlights the critical role of InlC as a key virulence factor produced by *L. monocytogenes*, facilitating intestinal colonization by promoting attachment and invasion of this bacterium to the host epithelial cells. Our result is supported by the previous studies (24, 25), which showed the InlC involvement in stabilizing the invasion process and enhances Listeria's capacity to establish infections in the intestinal lining, potentially leading to listeriosis and severe systemic infections if left untreated.

Additionally, the result of this research indicated that the InlC protein plays a significant role in promoting the spread, survival, and multiplication of *L. monocytogenes* within the epithelial cells. This conclusion is also substantiated by several investigations where they have been reported that InlC

interacts with human scaffolding protein Tuba or the host exocyst complex and this interaction helps control cortical tension and exocytic membrane trafficking, facilitating the creation of plasma membrane protrusions (10, 26). These protrusions, in turn, aid in the spread of *L. monocytogenes* from infected epithelial cells to neighboring cells. In addition, it was previously reported that the expression of InlC increases when *L. monocytogenes* escapes from the phagosome of mammalian cells (9). These combined findings suggest that InlC interactions with the host cellular machinery play a pivotal role in the survival, proliferation, and dissemination of *L. monocytogenes* within host cells.

Furthermore, the current findings indicated a significant up-regulation of exocyst in the rabbit intestinal tissues injected by *L. monocytogenes*, displaying a fold change of (2.3436), in contrast to the exocyst expression in the rabbit intestinal tissues injected by (PBS), where the fold change stood at (1.0461) (p-value 0.017). This result agreed with the recent finding reported that exocyst plays a critical role in *Listeria monocytogenes* infection response as it was significantly expressed (24). The exocyst complex plays a significant role in regulating the tethering and docking of vesicles at specific target membranes during various cellular processes, including exocytosis and membrane trafficking. It was also previously reported that exocyst has been implicated in this process by facilitating the delivery of Listeria-containing vacuoles to specific regions of the host cell membrane, thereby promoting bacterial uptake (24, 27). These findings align with our study as during infection, *Listeria* exploits host cell machinery to promote its entry into non-phagocytic cells, such as epithelial cells.

Conclusion:

Our study concluded that the increased expression levels of InlC enables *L. monocytogenes* to enhance its survival and spread within the intestinal host cells. InlC protein plays a very important role in manipulating cellular processes and also interfering with the host cell's defense mechanisms, allowing the bacterium to evade immune responses. Last but not least, the concurrent increase in the expression levels of exocyst suggests its involvement in cellular processes triggered by InlC, potentially contributing to the success of *L. monocytogenes* infection**.**

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Nil.

Ethical approval

The study was conducted under a protocol approved by a local ethics committee at Al-Qasim Green University, College of Biotechnology, Department of Medical Biotechnology, and Babel Health Directorate under the refrence number 935 dated on July 7, 2022.

Availability of Data and Materials

The data sets used and/or analyzed during the current study are available from the corresponding authors on reasonable request

Conflict of Interest

We declare that we have no conflict of interest.

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