Mechanism of infection of a human isolate \textit{Salmonella} (3,10:r:-) in chicken ileum: Ultrastructural study

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Background & objectives: Originally isolated from severe human food-poisoning cases, \textit{Salmonella} (3,10:r:-), a monophasic variety of otherwise diphasic serotypes such as \textit{S. weltevreden} and \textit{S. simi}, causes serious infections in man, animals and poultry. Mechanism of infection of this versatile and deadly organism is important to understand for its control. The objective of this study was to enhance our understanding of infection of \textit{Salmonella} (3,10:r:-) \textit{in vivo} at cellular level.

Methods: Aliquots of $10^9$ cfu of \textit{Salmonella} (3,10:r:-) organisms were injected intra-ileally in 24 h pre-fasted 3 month old broiler chickens by standard ligated ileal loop method. After 18 h, the fluid accumulated in the ileum was drained and small tissue pieces were fixed in 2.5 per cent buffered (pH 7) glutaraldehyde and subsequently in 1 per cent aqueous osmium tetraoxide. Ultra-thin sections of araldite-embedded tissue pieces were examined under transmission electron microscope operated at 100 KV after staining with uranyl acetate and lead citrate.

Results: Over 70 per cent of salmonellae interacting within 300 nm with ileal epithelial cells developed numerous surface blebs of periplasmic extensions designated “periplasmic organelles” (POs). Large sized POs were apparently pinched off as outer membrane vesicles (OMVs), 50-90 nm in diameter. Type III secretion needle complex-like “rivet complexes” (RCs) were viewed to rivet the bacterial outer and inner membranes together, allowing only pockets of periplasm to expand/inflate in order to liberate OMVs. Many OMVs were found visibly docked on the plasma membrane of host epithelial cells. The invading organisms appeared to leave the epithelial cells so as to find entry into the lymphatic vessels, where, they again appeared to be closely interacting with ileal macrophages, by forming numerous POs and concomitantly liberating OMVs. Inside the cytoplasm of macrophages, numerous tight phagosomes were seen, each containing two organisms. The final stage appeared to contain replicated salmonellae, four in each loose phagosome and, at the same time, macrophages also showed signs of apoptotic disintegration, culminating in the release of replicated salmonellae.

Interpretation & conclusions: Outer membrane vesicles released from a fiercely virulent human isolate, \textit{Salmonella} 3,10:r:- pathogens have been implicated in translocating biochemical signals from the host-interactive organisms to the eukaryotic cells at both stages of invasion leading to epithelial cell and macrophage infection \textit{in vivo}, in the chicken ileal model. A comprehensive cellular mechanism at ultrastructural level is outlined for typhoid-like \textit{Salmonella} infections caused by this humans-infecting organism.

Key words: Chicken ileum - human isolate - invasion & infection - outer membrane vesicles - \textit{Salmonella} (3,10:r:-) - type III secretion - ultrastructure
Salmonellae are responsible for causing food-poisoning problems and typhoid-like infections in humans and animals, resulting in huge monetary losses due to morbidity-linked reduction in productivity and increased costs of disease treatment/management. Therefore, it is important to understand their mechanisms of infection at cellular and molecular levels in order to innovate necessary interventions. *Salmonella* (3,10:r:-), originally isolated from severe human food poisoning cases, is capable of infecting animals and poultry. Interest in structural aspects of virulence dates back to the year 1992 from this laboratory when it was first demonstrated by transmission electron microscopy (TEM) that *Salmonella* (3,10:r:-) organisms located in close proximity of host cells and interacting in vivo with microvilli of epithelium in chicken ileum, developed numerous bacterial outer membrane bound surface appendages or periplasmic protrusions filled with bacterial secretions. Similar surface appendages (named as invasomes) were also later observed on *Salmonella* Typhimurium while closely interacting in vitro with cultured MDCK epithelial cells, as studied by scanning electron microscopy. Further TEM studies suggested that larger periplasmic protrusions could pinch off as 50-90 nm diameter sized bacterial outer membrane vesicles (OMVs), which could also be seen in fusion-like membrane-to-membrane contact with microvilli of host epithelial cells. Translocation of bacterial 'toxic' secretions from invading Gram-negative pathogens into host/target cells was lauded as discovery of a novel process *viz.*, vesicular exocytosis in prokaryotes and was considered to add a new structural dimension of type III secretion system. Later, these OMVs have also been associated with damage caused to eukaryotic host cells and aiding in invasion of the pathogens. It is now becoming increasingly clear that Gram-negative pathogens employ OMVs for targeting toxin delivery into mammalian cells and that they contain a variety of bacterial toxins and pathogenicity and also in cytokine production by neutrophils, besides acting as potent inducers of platelet aggregation. Recently, OMV release has also been shown to correlate directly with level of protein accumulation in the cell envelope which has been opined to represent a physiological stress response. The present study reports a comprehensive ultrastructural mechanism for invasion and infection of chicken ileum by the human isolate *Salmonella* 3,10:r:- organisms, with crucial role assigned to OMV's.

### Material & Methods

The pathogen, *Salmonella* 3,10:r:- (a monophasic variety of otherwise diphasic serotypes such as *S. weltvreden* and *S. simi*), originally isolated from human food poisoning cases, was obtained from National *Salmonella* Centre at the Indian Veterinary Research Institute, Bareilly, Uttar Pradesh. This particular strain has been typed distinctly from *S. weltvreden* and is maintained at this Centre. Cultures of the organisms originally isolated from cases of human food poisoning with 10⁹ cfu were injected into chicken ileum in 24 h pre-fasted 3 month old five broiler birds (obtained from Central Avian Research Institute, Bareilly and specifically maintained under suitable laboratory conditions), using standard ligated ileal loop methodology. The procedures used employ thorough washing-out of the ileum of its contents (including naturally inhabiting organisms). Another saving factor in this procedure was that the experimental (injected with the dose of organisms) and control ileal loops (injected with sterile medium without organisms) were located in the same animal(s) so as to allow for excellent control versus experimental sampling. The fluid was found to be exsorbed only in the experimental loops after 18 h of injection, and their contents were tested for presence of injected organism to make sure that reaction was indeed caused by *Salmonella* (3,10:r:-) pathogens. The fluid exsorbed/accumulated in the ileal loops was drained and ileal tissue pieces (size approximately 1-2 mm³) of the experimental and control loops were fixed in 2.5 per cent glutaraldehyde in phosphate buffer (pH 7) at 5°C for 6 h and subsequently post-fixed/block-stained in aqueous 1 per cent osmium tetroxide for 6 h at room temperature following standard methodology. The fixed tissue blocks were made in araldite and ultra-thin sections (approximately 500 Å in thickness) were cut using glass knife with an ultramicrotome (LKB Ulrotome III, Sweden). Ultra-thin sections obtained on 3 mm diameter copper grids, were stained with uranyl acetate and lead citrate stains for contrast and examined under JEOL JEM 1200EX electron microscope (Japan) working in transmission mode operated at 100 kilovolts. The electron micrographs were interpreted in detail and arranged in an order proposed to be a workable sequence of the progress of invasion and infection in vivo.

### Results

From intensive TEM studies and detailed interpretation of electron micrographs, a comprehensive
Fig. 1. An ultrastuctural mechanism of invasion and infection of a human pathogen *Salmonella* 3,10:α- studied in experimental infection of chicken ileum. A proposed sequence of steps A-I may lead to systemic infection. Inset is the detailed structure of a large-sized periplasmic organelle filled with secretory materials packed within a markedly inflated periplasmic space (PS), prior to its proposed liberation as an outer membrane vesicle (OMV). Fig. 1 A represents the organisms located near the center of the ileal lumen, which are not closely apposed to the host epithelial cells and thus considered to be non-interactive organisms. Fig. 1B represents the organisms closely interacting with ileal epithelial cell microvilli (mv) revealing numerous pockets of protruding periplasm, designated periplasmic organella. Fig. 1C represents salmonellae (sal) liberating bacterial outer membrane-bounded 50-90 nm diameter vesicles (MV) pinched off from large periplasmic organelle. These OMVs appear to dock on the microvillous membrane and a fusion-pore, supposedly formed at the contact point, is proposed to translocate the biochemical signals of the pathogen into host cytosol. Fig. 1D represents host ileal epithelial cells which have undergone cytoplasmic reorganization and membrane ruffle (R) formation after focal disruption of microvilli (curved arrow) that allow macro-pinocytosis (straight arrows) of the closely approaching salmonellae (S). This process is proposed to be signaled by contents of outer membrane vesicles translocated into the host eukaryotic cell cytosol. Fig. 1E represents salmonellae that pass through ‘corridors’ created in the ruffled epithelial cells (Fig. 1D) and then travel through lymphatic vessels of infected ileum (see Fig. 2) so as to come in contact with macrophages. Numerous outer membrane vesicles (MV) once again liberated by pinching off periplasmic organelles (p) are apparently taken up (curved arrows) by macrophages (M). This uptake is assumed to signal the macrophages to go into a stimulated phagocytosis mode. Fig. 1F represents the process of stimulated multiple-phagocytic cup (p) formation, helping engulf the approaching salmonellae (sal). Fig. 1G represents the mode of engulfment of pairs of organisms (A & B) and getting enclosed in single phagosomes (P). Fig. 1H represents a stage where macrophages may end up getting packed with numerous electron-opaque phagosomes in their cytoplasm, mostly not-fusing with co-incident lysosomes (L). Fig. 1I. represents the proposed last stage of the cycle at which replicated salmonellae, four in each loosened phagosome, appear to be released from disintegrating infected macrophages. Movement of such macrophages may result into systemic spread of the pathogens to lead to typhoid-like salmonella complications.
cellular mechanism for invasion and infection of chicken ileum by a human pathogen, *Salmonella* 3,10:r:- is proposed (Fig. 1). An amorphous surface ultrastructure was observed for most of the organisms located near the center of the ileal lumen when the organisms were located more that 2000 nm away from the epithelial cell microvilli (Fig. 1A). Such a surface morphology has therefore, been considered to represent a non-interactive (with host cells) state of the organisms. Organisms could be observed only in the tissue taken from ileal loops injected with live organisms and not in control loops given placebo. Marked surface changes were seen in organisms representing a majority (over 70%) of organisms located closely (within 300 nm) to epithelial cell microvilli in the ilial loops injected live organisms (Fig. 1B). Numerous bacterial outer membrane-bound periplasmic extensions/protusions were observed on these organisms, considered to represent a host cell-interactive state of the organisms. As these structures were present all around the surface of the organisms, they might show up as thin and long appendages or as fat ‘blisters’ which appear to signify a structural expression of acquisition of a virulent state during close cross-talk with host eukaryotic cells. These structures have been designated ‘periplasmic organelles’.

Fig. 1C showed 50-90 Å diameter size bacterial outer membrane vesicles (OMVs) apparently pinched off from large periplasmic organelles. Many of these OMVs appear to form intimate membrane-to-membrane contact between vesicular surface and host epithelial cell microvillous plasma membrane. As the OMV membrane should consist mainly of lipopolysaccharide (LPS) and host cell plasma membrane mainly of phospholipids, any fusion between them is likely to be assisted by some specialized proteins/receptors. *Salmonella* invasion proteins SipB and SipC have been shown to be present on OMVs (Hayward RD, University of Cambridge, England, personal communicaton). This may help in the generation of pore between OMV and host cell membranes, thereby, help translocating the OMV contents into the host cell cytosol &/or direct endocytosis of OMV’s as such.

A representative *Salmonella* organism (S) is shown to be located face-to-face with a ruffled host epithelial cell membrane (R) (Fig. 1D). A focal disruption of microvilli and reorganization of host cell cytoskeleton were markedly clear. The arrows suggest a possible path for intra-cytoplasmic entry of the organisms explaining the likely macropinocytosis process. Our results also showed that the surface appendages, referred to as periplasmic organelles were not observed on the salmonellae located close to the ruffled host cells. The observed loss of surface appendages at time of intracytoplasmic entry of salmonellae could be explained as prior liberation of OMVs from inflating periplasmic organellae here as shown in Fig. 1C. It appears that the invading *Salmonella* 3,10:r:- pathogens use the ileal lining epithelial cells as a safe corridor to get entry into the inner regions of the ileum. Fig. 2 shows these organisms passing through lymphatic vessels of chicken epithelium. Here, these organisms were observed as being abundantly phagocytosed by macrophages.

A representative salmonella organism is shown to be located closely face-to-face with a macrophage in the lymphatic vessel of chicken ileum (Fig. 1E). Interestingly, once again, the *Salmonella* 3,10:r:- pathogens (arrow-heads) interacting with macrophages while passing through the lumen of lymphatic vessels (thick bold arrows) in experimentally infected chicken ileum (P, phagosome; pc, phagocytic cup).

![Fig. 2. *Salmonella* 3,10:r:- pathogens (arrow-heads) interacting with macrophages while passing through the lumen of lymphatic vessels (thick bold arrows) in experimentally infected chicken ileum (P, phagosome; pc, phagocytic cup).](image-url)
organisms located in similar orientations showed numerous periplasmic organelle on their surface, coincident with several OMVs liberated therefrom, coming in close contact with the plasma membrane of macrophages. Many OMVs were also seen located inside the peripheral regions of the cytoplasm of these macrophages on the portions closely interacting with OMVs. Therefore, a plausible explanation is that these OMVs are engulfed/endocytosed by the macrophages, thereby translocating bacterial vesicular contents into the cytosol of macrophages. Besides the contents of the OMVs, their membrane lipopolysaccharide and outer membrane proteins are also taken inside the macrophages. In case of Salmonella 3,10:r:- infection of chicken ileum as reported here, the observations suggest that OMV uptake/engulfment triggered the macrophages into invigorated activity of phagocytosis of numerous organisms. This became obvious from numerous phagocytic cup formations around the closely approaching salmonellae (Fig.1F) and their entrapment in pairs in tight phagosomes (Fig.1G). Numerous tight phagosomes containing these organisms predominated the scene of such macrophages, which also appeared not to fuse with close-by located lysosomes in the macrophage cytoplasm (Fig.1H). Many infected macrophages also appeared to be undergoing apoptotic disintegration, concomitantly showing numerous loose phagosomes containing four organisms each (Fig. II). This stage of macrophages showed replicated salmonellae, being let out for re-infection, due to disintegration of the infected macrophages.

Discussion

With over 2323 known serotypes, Salmonella infections which occur commonly in man, animals and birds the worldover, perpetually take a heavy toll in the form of morbidity-linked losses besides thrusting heavy expenditures on management and treatment of the disease. The serotype, Salmonella 3,10:r:- is responsible for causing severe food-poisoning infections in human beings and animals. In the chicken model of experimental infection studied in vivo, interesting ultrastructural changes were observed in both the interacting pathogens and the eukaryotic host cells. Host-pathogen interactions encountered in Gram-negative organisms at close interface with eukaryotic cells have been recently linked with the bacterial type III secretion system (T3SS) and OMV-associated export of bacterial toxins. The Gram-negative pathogens employ T3SS for translocation of a cocktail of bacterial effector proteins and virulence determinants from the organisms to directly into host cell cytosol. Spatio-temporal regulation of these effectors accomplishes fine-tuned modulation of host cell machinery. Such hijacking of eukaryotic functions is not only accomplished by intracellular bacterial pathogens, but a similar subversion of host cell actin dynamics is also achieved by extracellularly infecting enteropathogens like enteropathogenic and enterohaemorrhagic Escherichia coli.

Our findings showed that periplasm of Salmonella 3,10:r:- organisms located in close proximity (within 300 nm) of host ileal epithelial cells as well as tissue macrophages played a significant role at the host-pathogen interface. It was earlier proposed that signals from host cells like antimicrobial peptides may induce synthesis of bacterial toxins. It has recently been shown that antimicrobial peptides do trigger pathogen virulence as the two-component regulatory system PhoP-PhoQ of Salmonella is activated by binding to antimicrobial peptides, thereby promoting gene transcription necessary of Salmonella survival within the host. It was therefore opined that antimicrobial peptides might act as a double-edged sword, promoting antibacterial immunity while simultaneously triggering pathogen virulence.

The tightly packed LPS molecules in the bacterial outer membrane are the first barrier to antimicrobial peptides. Further, only the killer form of antimicrobial peptide penetrates the lipopolysaccharide layer and induces LPS micellization. Virulence proteins and allied determinants may be quickly synthesized and transported across the bacterial cell membrane into the periplasmic space via the general secretory pathway under influence of suitable inducers like change in temperature, pH and chemical composition in the microenvironment around the eukaryotic host cells. Indirect evidence on E. coli suggests that H+-ATPase machinery uses proton motive force to generate ATP which, in turn, is essential for protein translocation via OMVs. Numerous pockets of protruding periplasm (designated as periplasmic organelles for their being physiologically significant structures) were observed all around the Salmonella 3,10:r:- organisms approaching closely and interacting with host epithelial cells or macrophages. A model for molecular structure of the periplasmic organelle has already been proposed and these organelles have been explained to represent a secretion-active virulent state of the organisms, ready to secrete the bacterial toxins and secretory products as OMVs.
OMVs liberated from the secretion-active *Salmonella* 3,10:r:-, have been proposed to be released by pinching off inflated periplasmic organelles filled with bacterial toxins and exoproteins secreted by the general secretory pathway (GSP)\(^1\). It was proposed that fusion of OMVs with the host epithelial membrane may result in the translocation of bacterial enterotoxins to directly inside the host epithelial cells\(^2\). A similar process for the release of heat-labile enterotoxin via general secretory pathway as OMVs has been observed for *E. coli* and further a mechanism of OMV-mediated receptor-dependent delivery of bacterial toxin into host cells was implicated\(^3\). Similarly, Shiga toxin was also found to be released as OMVs from periplasmic space of *Shigella dysenteriae* and that the secretion was induced by an antimicrobial compound, mitomycin C\(^4\). On the same pattern, OMVs containing vacuolating cytotoxin (VacA), which was immuno-localized in the periplasm and outer membrane of intact *Helicobacter pylori* bacteria, appeared to originate from blebs arising on the bacterial outer membrane. Both soluble secreted VacA and VacA-containing OMVs were internalized by MKN28 cells and were detectable in the gastric mucosa of *H. pylori*-infected humans\(^5\). Likewise, active cytotoxic necrotizing factor 1 (CNF1) secreted from uropathogenic *E. coli* has also been found to be associated with OMVs thereby suggesting that CNF1 is transported to the environment of the infected tissue via OMVs\(^6\). The salmonella invasion protein SipB of T3SS was shown to direct heterotypic membrane fusion, allowing delivery of contents from *E. coli*-derived liposomes into cytosol of living mammalian cells\(^7\). Such a mode of translocation of bacterial secretions as OMVs into another host/recipient cell has, therefore, been described as “vesicular exocytosis from prokaryotes”\(^8\). As earlier, the exocytosis process was traditionally associated only with eukaryotes\(^9,10\). OMVs have also been linked to type I\(^1\) and type III\(^11,12\) secretory systems of Gram-negative organisms.

In the ileal epithelial cells showing ruffled membrane and cytoskeletal reorganization of the cytoplasm, it was notable that organisms at the surface of the ruffled site did not reveal any periplasmic organelles on their exterior. This may be explained as these organelles have already been pinched off as OMVs, which in turn have seemingly accomplished their task of translocating the bacterial virulence determinants into the interacting ileal epithelial cells. It has been proposed that SipB (located in/on OMVs) secreted by the invading *Salmonella* triggers bacterial entry into eukaryotic cells and this is blocked by a SipB-derived polypeptide\(^13,14\). Manipulation of the host cell actin cytoskeleton by *Salmonella enterica* for entry into epithelial cells has been extensively studied\(^15\). Our study suggests that *Salmonella* 3,10:r:- organisms created corridors via the ruffled locations in order to get access to inner sites in the ileum, as the organisms were seen in the lymphatic vessels where they were observed to closely interact with macrophages (Figs. 1 & 2).

*Salmonella* 3,10:r:- organisms closely interacting with macrophages, developed on their surface, large blebs (periplasmic organelles), which appeared to liberate numerous OMVs, which in turn, were apparently taken up by the macrophages. This process has been assigned the task of translocation of biochemical signals including LPS from the invading pathogens to directly into eukaryotic host cells, macrophages, at this stage. This led to augmented phagocytic cup formation and consequent engulfment of organisms into the macrophage cytoplasm (Fig. 1). Modulation of leukocyte response mediated by other Gram-negative pathogens has also been reported. Recently, OMVs of *Neisseria meningitidis* have been shown to activate monocytes in an LBP-, CD14- and TLR4/MD-2-dependent fashion with pro-inflammatory effect\(^16\). Also, OMV-mediated modulation of leukocyte adhesion molecule expression and increased reactive oxygen species (ROS) production is likely to increase entrapment of leukocytes in the microcirculation and contribute to untoward inflammatory reactions as noticed in systemic meningococcal disease\(^17\). Another report\(^18\) shows that OMVs containing cytotoxic necrotizing factor 1 (CNF1), but not purified CNF1, act in a dose dependent manner, on polymorphonuclear leukocytes to attenuate their antimicrobial activity. This study reveals that OMVs provide a means for delivery of CNF1 from uropathogenic *E. coli* to these host cells, and thus negatively affect the efficacy of acute inflammatory response to these pathogens. Further, CD\(^+\) T cells and toll-like receptors recognize *Salmonella* antigens expressed in bacterial surface organelles including OMVs. Thus, genetically co-ordinated surface modifications may provide a growth advantage for *Salmonella* in host tissues by limiting both innate and adaptive immune recognition\(^19\). OMVs generated by *H. pylori* bear serologically recognizable Lewis antigens, which may contribute to the chronic immune stimulation of the host. The ability of these OMVs to absorb anti-Lewis auto-antibodies suggest that they may, in part, play some role in putative autoimmune aspects of *H. pylori* pathogenesis\(^20\).
Fig. 1-G shows that two organisms entering at one location of the macrophages get enclosed in one tight phagosome. Numerous tight phagosomes are observed to occupy the bulk part of the cytoplasm of macrophages, where they appear to be resistant to fusion with lysosomes, located nearby (Fig. 1H). Individual organisms are not really discernible in the tight phagosomes due highly electron-dense contents. However, in the spacious phagosomes, four organisms are clearly visible in each phagosome (Fig. 11). Thus, it suggests that two organisms originally entrapped in one tight phagosome replicate into four organisms coincident with loosening of the tight phagosomes into spacious ones. Parallel apoptotic disintegration of infected macrophages appears to release the replicated pathogens in body of the host, promoting infection of more host cells. Circulation of infected macrophages may be envisaged to lead to systemic infection. Recently, it has been shown\(^5\) that OMVs of \textit{H. pylori} induce apoptosis in gastric epithelial cells. Further, this apoptosis is not mediated by mitochondrial pathway as is demonstrated by the lack of cytochrome c release with the activation of caspase 8 and 3.

Overall, this study indicates an important role played by OMVs released by \textit{Salmonella} 3,10:r:- pathogens at both stages of invasion that is of epithelial as well as macrophage cells. An earlier study revealed that protein translocation into OMVs required ATP and the proton motive force might also contribute but appear not to be essential in \textit{E. coli}\(^4\). It is plausible to opine that proton motive force may generate ATP with the help of H\(^+\)-ATPase, and, in turn, ATP may be utilized for transporting proteins across the cell membrane into the periplasmic organelles to be eventually released as OMVs. The specialized T3S assembly of pathogenic and symbiotic Gram-negative bacteria comprises a multi-protein transmembrane complex and an ATPase homologous to F1-ATPase beta-subunit, which forms a double hexameric ring assembly in the inner membrane as studied for HrcN of \textit{P. syringae}\(^5\). Early, a mechanism was proposed in which OMVs are formed when the outer membrane expands faster than the underlying peptidoglycan layer\(^6\). Recently, it was shown that OMV production by \textit{E. coli} is independent of membrane instability (detergent-sensitivity, leakiness) but, gene disruption, however, can cause under or over-production (5 to 200-fold increase) of OMVs, relative to wild type levels\(^5\). Nonetheless, gene activation leading to synthesis of virulence proteins under induction from antimicrobial peptides (present in the microenvironment or those secreted by eukaryotic host cells) have been postulated to cause augmented secretion of OMVs containing secretory exoproteins\(^8\). Experimental proof of this viewpoint has been recently obtained\(^4\) confirming that antimicrobial peptides actually trigger pathogen virulence by binding to Phop-PhoQ regulatory system of \textit{Salmonella}. Of late, role T3S needle complex-like assembly has been implicated in the release of OMVs in the analogy of blowing off soap bubbles with the formation of tube-like assembly T3S needle/rivet complexes\(^3\). Although, confirmatory proof for the existence of a generalized OMV model for T3SS\(^3\) is still awaited, yet it does obviate many unanswered questions posed to popular \textit{injectisome} model\(^8\) on the \textit{modus operandi} of translocation of semi- or folded proteins through a rather narrow and long conduit of the T3SS assembly. Interestingly, OMVs have been recently shown to release type I secreted alpha-haemolysin\(^21\) from \textit{E. coli}. Also, some T3SS proteins like SipB & SipC of \textit{Salmonella} have been shown to be associated with OMVs. This important ultrastructural study is hence envisaged to stimulate further work using monoclonal antibodies and allied techniques to immunolocalize type I, III and other secretory proteins in the OMVs of Gram-negative pathogens, under \textit{in vitro} and \textit{in vivo} conditions. To would establish their role in host-pathogen interactions, inter-species competition of pathogens, and intercellular communication within bacterial colonies & inter-kingdom singaling.

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