Phenotypic Characterization of Auxotrophic Mutant of Nontyphoidal Salmonella and Determination of Its Cytotoxicity, Tumor Inhibiting Cytokine Gene Expression in Cell Line Models

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Abstract

An auxotrophic mutant of non-typhoidal *Salmonella* (NTS) strain was phenotypically characterized in this study. The characterization was based on phenotype, morphology, motility, biofilm forming ability, growth kinetics, etc. The phenotypic results from the above experiments determined that the mutant showed variation in phenotypic characters from that of wild-type strain. Subsequently, mutant and wild-type NTS were subjected to epithelial cell invasion and intracellular replication assays. The real time PCR analysis was also performed to analyse expression of tumor inhibiting cytokine genes and virulence genes post bacterial infection in cell lines. The mutant showed highest invasion potential than wild-type NTS whereas the replication of mutant was slower in both the cell lines. Similar to the wild-type strain, the mutant also retained the cytotoxic potential when analysed *in vitro*. Furthermore, the expression of proinflammatory cytokine genes such as *TNF- α* and *IL-1 β* was upsurged with the downregulation of anti-inflammatory cytokine genes like *TGF- β* , *IL-6* and *IL-10* post infection of the mutant strain in cell lines. In addition, virulence genes of *Salmonella* pathogenicity island one and two of mutant were downregulated *in vitro* except *invA* in HeLa cell line. Therefore, the auxotrophic mutant showed positive attributes of a potential antitumor agent in terms of expressing tumor inhibiting cytokine genes when assessed *in vitro*. Though the study did not check the tumor inhibitory effect of NTS strain directly, findings of the study emphasizes on the development of a novel strain of NTS with less virulence and more immunogenic traits to inhibit tumor cells.

Introduction

Cancer is a multifactorial disease causing high morbidity and mortality worldwide every year. The diverse factors that are associated with the development of certain cancers include genetic background, age, diet, smoking, exposure to pollutants, alcohol consumption, body weight, microbial infection etc., leading to global cancer mortality (Ashu et al. 2019). Due to the lack of effectiveness and inadequate tumor penetration in conventional therapies like chemotherapy, radiation therapy etc., cancer therapy has not gained much success (Khawar et al. 2015). This has turned the attention to look for better alternative therapeutic strategies. Therefore, the immunotherapeutic approach of tumor treatment was explored which thereby proved novel wherein it escalated the effectiveness of identifying and selectively mounting on the tumor cells to produce antitumor response by sparing the surrounding healthy tissues (Nagarsheth et al. 2017). The immunotherapies such as checkpoint inhibitors, monoclonal antibodies, anti-cytotoxic T-Lymphocyte associated protein 4 (CTL-4) and programmed death protein 1 (PD1) targeted immunotherapy are being tried in clinical trial (Torres et al. 2018; Scheiner et al. 2019) but was not successful as anti-oncolysates because it reduced the immune tolerance capacity due to its systemic administration. This has prompted the use of an alternative therapy such as bacteria mediated cancer therapy to cause cancer cell destruction. The practice of bacterial application targeting tumor cells dates back to the age of William B. Coley, an American oncologist who observed the role of bacteria in demolishing tumor in patients with acute bacterial infections in the late 19th century (Kramer et al. 2018). Coley's work didn't gain adequate attention due to the incomplete understanding of the mechanism behind its tumor regression. Hence, the focus was shifted to radiation therapy and chemotherapy to destroy tumors. Nevertheless, these strategies didn't succeed in eliminating tumor completely and it resulted in severe side effects (Jazeela et al. 2020). Thereafter, the bacterial mediated tumor therapy renewed and many different bacterial species with immunogenic and anticancer potential are currently being explored. So far, the oncolytic nature of various bacterial strains such as *Mycobacterium bovis*, *Listeria monocytogenes*, *Clostridium*,

Salmonella Typhimurium etc., have been explored *in vivo* (Adkins et al. 2012) due to their distinctly colonizing and rapid proliferating nature in the tumor microenvironment. Amongst these, *S. Typhimurium* was extensively studied *in vitro* where it showed effective invasion and destruction of broad range of cancer cell types and *in vivo* in oxygenic and anoxygenic tumor regions (Hoffman 2016). Thus, this bacterium was regarded as the master strain in the experimental cancer therapy analysed till date. The *S. Typhimurium* VNP20009 $\Delta msbB/\Delta purI$ (lipid A and purine auxotrophic mutant) is one among various mutants of *S. Typhimurium* which was taken to phase 1 clinical trial after it was tested for its efficacy in mice and swine; but it resulted in treatment failure due to over attenuation (Toso et al. 2002) and also because of its toxicity in bone marrow, spleen and liver when injected intra-tumorally (Mercado-Lubo and McCormick 2017). According to Zhang *et al.* other mutants of the *S. Typhimurium* like A1-R auxotrophic mutant, proved to be more potent antitumor agent when compared to VNP20009 strain and showed inhibition of Lewis lung carcinoma (Zhang et al. 2015). In mouse models, monotherapy of A1-R auxotrophic mutant was effective against primary and metastatic human prostate, breast, and pancreatic cancer in addition to osteosarcoma, fibrosarcoma, and glioma (Hoffman 2016). A1-R also showed positive attributes towards destruction of tumors with high vascularity (Liu et al., 2010) and it encouraged stem like and non-stem cancer-cell death *in vivo*, indicating that chemo-resistant cancer stem-like cells could be lysed by A1-R mutant (Hiroshima et al. 2013). Another mutant of *S. Typhimurium* defective in the ppGpp synthesis ($\Delta ppGpp$: depletion of *relA* and *spoT*) exhibited the up regulation of certain tumor suppressing cytokine genes like *IL-1 β* , *IL-18* and *TNF- α* and also triggered the production of NLRP3, IPAF (inflammasome coding genes) in MC38 and CT26 in colon cancer murine models (Zheng et al. 2017). *S. Typhimurium aroA-aroD* double mutant (harbouring the Flt3 Ligand) is a genetically-engineered strain which was targeted against melanoma in mice model and it showed 50% tumor suppression but soon resulted in low fitness within the tumor and this strain was regarded as the Class I mutants (Arrach et al. 2010) which was less potent than Class 2 mutants in suppressing tumor growth. The anticancer property shown by *S. Typhimurium* is also elicited by the passive instigation of the infiltrating pro-inflammatory cytokines upon its injection *via* intravenous route (Crull et al. 2011). Tumor lysis was observed when the pro-inflammatory cytokines like *IL-1 β* and *TNF- α* were released post *Salmonella* infection where in apoptosis-induced tumour cell death was shown by *TNF- α* and both helper and cytotoxic T-cell induced tumour cell death was exhibited by *IL-1 β* (Kim et al. 2015).

Over the past several years, different mechanisms of bacteria-tumor interactions in addition to conventional antitumor therapies were studied *in vitro* and *in vivo* to successfully eradicate cancer cells but the results were unreliable. Therefore, it is always desirable to use less virulent serovar of *Salmonella* in order to reduce the toxic effects to healthy cells. Hence, in this study an auxotrophic mutant of a non-typhoidal *Salmonella* (NTS) serovar, *Salmonella* Oslo generated in our previous study was characterised and explored for its role in triggering the tumor suppressing cytokine genes in cell line models.

Materials And Methods

Bacterial strains

An auxotrophic mutant of *Salmonella* Oslo generated in our previous study (Jazeela et al. 2020) was further characterised phenotypically in this study. The wild-type strain of NTS was used as the control strain for all the experiments undertaken in this study.

Screening of auxotrophic mutant for the variation

Phenotypic characteristics

Phenotypic confirmation of the suspected transformants along with the wild-type bacteria as a positive control were done by streaking the cultures on minimal agar medium (HiMedia, Laboratories Pvt. Ltd., India) supplemented with and without amino acids leucine and arginine. A non-selective agar media, Luria-Bertani agar (LBA) was also used as an enriched media to check for the growth of the transformants as master plate. The strains were also streaked on selective and differential agar plates of *Salmonella* such as Xylose Lysine Deoxycholate (XLD) (HiMedia, Laboratories Pvt. Ltd., India) and Hektoen Enteric Agar (HEA) (HiMedia, Laboratories Pvt. Ltd., India) to differentiate the growth pattern (data not shown). After incubating them at 37 °C for sufficient period of time, the plates were observed for the growth of the mutants and compared it with the wild-type strain.

Morphological characteristics

The auxotrophic mutant strain obtained was checked for its morphological difference in LBA and was compared with the wild-type strain. Briefly, bacterial strains were revived in a 5ml LB broth medium and incubated at 37 °C until a mid-log phase culture is obtained. One ml of these cultures were centrifuged at 8000 x g for 5 min. The supernatant was discarded, and cell pellets were resuspended in 1ml of the PBS (HiMedia, Laboratories Pvt. Ltd., India) and it was serially diluted in different concentrations like 1:10, 1:100 etc. From each dilution, 100 µL of the diluted samples were plated on LBA and incubated at 37 °C for overnight growth. Plates were observed for the appearance of colonies after 24-48 h and the results were recorded.

Motility assay by soft agar stabbing

The motility of mutant and wild-type strain was checked by growing them in soft agar medium. Briefly, both the cultures were inoculated to soft agar containing 1% tryptone, 0.5% NaCl and 0.5% agar (wt/vol). The cells were incubated at 37 °C to an optical density of 0.6 OD₆₀₀. Inoculation of these cultures was done by using sterile straight needle by stabbing through the centre of the semisolid agar tubes approximately one-half the depth of the medium. These tubes were incubated at 37 °C for 4 h and observed for the growth of the culture. The incubation was continued for an additional day or more (24-48 h). The motility was observed based on the diffused growth produced by the strains.

Bacterial growth kinetics

The growth kinetics of both the wild-type and mutant strain was compared. Briefly, an overnight grown culture with 0.6 OD₆₀₀ was inoculated to a flask containing 200 ml LB broth to obtain an initial OD of 0.001 at zero hour. The flask was then incubated at 37 °C in a shaker incubator (New Brunswick, Eppendorf) at 200 rpm for varying period. The optical density was measured at 600 nm from 0-30 h using a spectrophotometer (Eppendorf BioPhotometer® D30, Germany). The experiments were performed in triplicates. The statistical differences between the growth of these strains were calculated by two-way ANOVA. A *p*-value less than 0.05 were considered as statistically significant.

Biofilm assay

Biofilm assay was performed and quantified in polystyrene microtiter plates (Eppendorf, Germany) (Stepanović et al. 2004). Briefly, 230 µL of trypticase soy broth (TSB) (HiMedia Laboratories Pvt. Ltd., India) was added to 96 well flat bottom polystyrene microtiter plates. About 20 µL of the overnight cultures of both wild-type and mutant strains were added to each well. The microtiter plates were incubated for 24 h at 37 °C. Following incubation period, the plate contents were discarded and washed thrice with sterile 1 x phosphate buffered saline (PBS) (HiMedia, Laboratories Pvt. Ltd., India). About 150 µL of methanol was added to fix the cells and 200 µL of 1% crystal violet to stain it. Post staining, 200 µL of glacial acetic acid (thirty three percent (v/v) was used to resolubilize. Then the plate was measured for its absorbance value at 630 nm (OD₆₃₀) in an ELISA reader (ELX 800, Bio-Tek Instrument, USA) and based on this result, bacterial strains used were classified as strong, moderate, weak and non-biofilm former. The experiments were performed in triplicates. True biofilm level was obtained after subtracting the optical density values of sterile control from the test values. The cut-off optical density was defined as 3 standard deviations above the mean O.D of the negative control (Bhowmick et al. 2011).

***In vitro* assays using wild-type and mutant strains**

Determination of epithelial cell invasion and intracellular replication in HEK-293 and HeLa cell lines using wild-type and mutant *Salmonella* Oslo strains

The HEK-293, RRID: CVCL_0045 and HeLa, RRID: CVCL_0030 cell lines were procured from National Centre for Cell Science (NCCS), Pune, India and preserved at -196 °C after passaging. These cell lines were then revived and plated into 24 well cell culture plates (Eppendorf, Germany) at 1.5×10^5 (HeLa/HEK-293 cells/well) and incubated for 24 h at 37 °C with 5% CO₂. Monolayer of cells with 70-80% confluence was selected for further experiment. The plates were washed with Dulbecco's phosphate buffered saline (D-PBS) (HiMedia, Laboratories Pvt. Ltd., India) prior to bacterial infection to remove the antibiotic content present in the pre-existing medium and then further supplied with the DMEM medium containing 10% Fetal Bovine Serum (FBS) w/o antibiotics. These plates were incubated at 37 °C until the OD₆₀₀ of the bacterial culture reached 0.6. Bacteria at the multiplicity of infection (m.o.i) of 10 were infected on to cell lines and epithelial cell invasion and intracellular replication were carried out (Blanc-Potard et al. 1997; Rakeman et al. 1999; Freeman et al. 2003) with minor modifications. Bacterial cells were counted by plating 100 µl of different dilutions on non-selective agar plates at 2 h and 16 h post infection. Epithelial cell invasion was assessed by the number of bacteria recovered at 2 h and intracellular replication was measured by the ratio of bacteria recovered at 16 h to the number of bacteria at 2 h. All the experiments were performed in triplicates. The data obtained as result of epithelial cell invasion and intracellular replication of bacterial strains in cell lines were subjected to unpaired Student's *t*-test with 5% significance level.

Determination of cytotoxicity assay in HEK-293 and HeLa cell lines using wild-type and mutant *Salmonella* strains

Evaluation of cytotoxicity caused by wild-type and mutant *Salmonella* strains in HeLa and HEK-293 cell lines was carried out from 0-24 hpi and compared the difference between the cytotoxic potential of these strains. Cell free supernatant of every time points were subjected to Lactate Dehydrogenase assay (LDH). LDH activity was determined as per manufacturer's protocol (Roche Applied Science; Indianapolis, IN) (Bhunia et al. 1994; Maldonado et al. 2005) which quantifies the amount of LDH release from the cell lines to the supernatant

upon cell lysis. Cell free supernatant from uninfected cell lines remained as the negative control. The reaction was read in an ELISA reader (ELX 800, Bio-Tekk Instrument, USA) to measure the absorbance of the sample at 490 nm using Gen5 software. The experiments were carried out in triplicates.

Infection of SO1 and LAT9 on epithelial cell lines and extraction of total RNA

Epithelial cell infection with the bacterial strains was carried out in T75 cell culture flasks (Eppendorf, Germany) according to the standard protocol (as mentioned earlier). Following bacterial infection, the T75 cell culture flasks were incubated for different time intervals *viz* 0 h, 2 h, 4 h, 6 h at 37 °C in an incubator supplied with 5 % CO₂. The growth medium was discarded following incubation period, and the epithelial cells were detached using 0.25% Trypsin-EDTA (HiMedia, Laboratories Pvt. Ltd., India) and the cells were collected in DMEM containing 10% FBS in 15 ml centrifuge tubes (Eppendorf, Germany). This cell suspension was centrifuged at 4 °C for 15-20 min at 4000 rpm to collect the cell pellet and it was subjected for RNA extraction using the RNeasy mini Kit (Qiagen, Germany) according to the manufacturer's instructions with minor modifications.

cDNA synthesis

The RNA samples were further subjected for DNase treatment (Thermo Fisher Scientific, USA) according to the manufacturer's guidelines to remove the remnant DNA. The quality and quantity of the RNA was measured using Nanophotometer (IMPLEN, Germany). The RNA samples obtained were normalised to one microgram for cDNA synthesis. The reverse transcription of the RNA samples was carried out using Reverse Transcription kit (Takara Bio USA, Inc.) according to the manufacturer's instructions. Gene expression was measured by using SsoFast EvaGreen Supermix (BioRad, USA) with minor modifications. Briefly, one microgram of each RNA samples with 50 µM of random hexamers were denatured at 65 °C for 5 min and added to the reaction mixture containing 5 X PrimeScript Buffer (50 mM Tris-HCl with pH 8.3, 375 mM KCl and 15 mM MgCl₂), 20 units of RNase inhibitor, 10 mM of dNTP mix and 100 units of PrimeScript reverse transcriptase. The reaction mixture was incubated at 42 °C for 60 min followed by heating at 72 °C for 10 min and then cooled to 4 °C. cDNA samples were stored at -20 °C until further use.

Expression of cytokine genes and virulence genes post bacterial infection in different cell lines by quantitative real time polymerase chain reaction (RT- qPCR)

The expression level of the cytokine genes (Table 1) expressed by the cell lines as a result of bacterial infection as well as the expression of bacterial virulence genes (Table 2) were quantified by qPCR at different time on HEK-293 and HeLa cell lines. Appropriate housekeeping genes such as *RP-II* (RNA polymerase II) for cytokine gene expression and *icdA* (Isocitrate dehydrogenase) for virulence gene expression were selected. The real-time PCR primers were designed using Primer3 software and optimum concentrations of primers were determined following standardization. The qPCR analysis was carried out using SsoAdvanced™ universal SYBR green Supermix (BioRad, USA) with minor modifications. The total reaction mixture (10 µl) consisted of 5 µl of advance 2X universal SYBR green super mix, 200 nM of forward and reverse primers for cytokine genes/100 nM of forward and reverse primers for virulence genes, and 1 µl of cDNA samples each and adjusted to 10 µl by adding nuclease free water (HiMedia Laboratories Pvt. Ltd., India). The reaction was performed in a real-time detection system (CFX96 Touch™, BioRad, USA) with cycling conditions as follows: initial denaturation at 95 °C for 3 min followed by 39 cycles of denaturation at 95 °C for 10 s, primer annealing temperature at 55 °C for 30 s

for cytokine genes and 45 cycles of denaturation at 95 °C for 10 s for virulence genes which had an annealing temperature of 54 °C for 20 s and elongation at 72 °C for 20 s. The amplification data was acquired by BioRad CFX Manager at the end of each elongation step. The expression of target genes was normalized to *RP-II* (housekeeping gene) during analysis of cytokine genes and *icdA* (housekeeping gene) when virulence gene expressions were analyzed respectively. The analysis of relative gene expression was carried as described previously (Livak and Schmittgen 2001). The data obtained from the real-time PCR were statistically analyzed by independent sample *t*-test with 5% significance level.

Statistical analysis

To calculate the statistical significance between two groups, experimental results were subjected to unpaired student's *t*-test using online statistical software GraphPad Prism v5.01 (<http://www.graphpad.com/quickcalcs/ttest1.cfm>) and two-way ANOVA was performed for more than two groups using SPSS software (version 16.0; SPSS, Chicago, IL, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

The auxotrophic mutant of the wild-type SO1 was named as LAT9. The mutant strain (LAT9) was characterized for the phenotypic variation exhibited from the wild-type strain and was checked for its attributes in cell line model system.

Phenotypic variation exhibited by the amino acid auxotrophs

The LAT9 strain did not grow in minimal media without any additional supplement of the amino acids. However, it showed slight growth on minimal media with amino acids supplements (Fig. 1). The mutant strain has shown good growth when grown on complete media which served as a control. These results further indicate that the mutant strain has become auxotrophic to certain growth requirements which are essential for its growth. The wild-type strain was served as the positive control.

Morphological variations in the mutant strain

LAT9 differed from SO1 when grown on non-selective agar medium. The colonies of SO1 appeared as circular and few were irregular, large, raised, and slimy in nature whereas the LAT9 appeared as small sized colonies. The experiment was performed in triplicates and the results were found to be persistent (Fig. 2).

Motility test

Motility was observed visually by diffused growth spreading from the line of inoculation. At 4 h post incubation, SO1 showed growth around the stab line whereas LAT9 did not show any growth. After an overnight incubation of both the tubes at 37 °C, SO1 showed diffused and thick growth throughout the medium while LAT9 diffused in an irregular fashion with less turbid growth (Fig. 3).

Growth kinetics

Growth kinetics of LAT9 was slower than SO1 and it was statistically significant with a p -value of >0.001 . The lag phase was >2 times than SO1 when evaluated under the similar culture conditions. The culture density of LAT9 was found to be lesser than that of SO1 when measured the absorbance at 600 nm spectrometrically until 30 h. Thus, the difference in the growth pattern was clearly observed among two strains (Fig. 4).

Detection of biofilm formation

The biofilm formation was assessed for both SO1 and LAT9. The former strain showed a strong biofilm formation (OD of 0.513) whereas the latter strain showed no biofilm formation (OD of 0.1). This result indicates that auxotrophic mutant has lost its biofilm forming ability. In addition, the calcoflour stained images showed that biofilm forming ability was hindered in LAT9 whereas SO1 displayed strong biofilm formation (Jazeela et al. 2020).

Epithelial cell invasion and intracellular replication assay

The epithelial cell invasion by LAT9 was markedly high (1.6-fold higher in HeLa and 3.3-fold higher in HEK-293 cell lines) when compared to the SO1. There was a significant difference (p -value > 0.001) between the strains in the bacterial count recovered at 2 hpi (Fig. 5a and 5b). In contrast to the invasion ability of LAT9 in epithelial cell lines, intracellular replication of LAT9 was evidently decreased and slower than SO1 (Fig. 5c and 5d). Invasion by the *Salmonella* strains (SO1 and LAT9) is necessary to cause infection since it is an intracellular bacterium. This data indicates that invasiveness is unaffected in auxotrophic mutant. Although the invasion of LAT9 was higher than SO1, reduction in the multiplication of the former strain in the epithelial cell lines may be due to the slow growth rate of LAT9.

Determination of cytotoxicity assay in HEK-293 and HeLa cell lines using SO1 and LAT9 strains

Overall, the cytotoxicity potential of both SO1 and LAT9 was higher in HEK-293 cell line than HeLa cell line. At 20 hpi, SO1 and LAT9 have shown 100 % cell death in HEK-293 cell line whereas, HeLa cells attained 100 % cell lysis at 22 hpi of SO1 and at 24 hpi of LAT9 respectively. In HEK-293 cell line, there was a gradual rise of cytotoxicity caused by SO1 from 0-16 h and a drastic increase in the percentage of cell death was seen from 16-18 h whereas in HeLa cell line, there was a variation in the trend of cell death percentage obtained from 0-18 hpi of both SO1 and LAT9 cells for unknown reasons. Additionally, there was an observable difference in the cytotoxicity produced by LAT9 at 6 hpi in HeLa cell line when compared to HEK-293 cell line.

Expression of cytokine genes *via* bacterial infection on epithelial cell lines

In HeLa cell line, an upsurge of *TNF- α* was observed post LAT9 infection at all the time points except at 2 hpi (Fig. S(a)) whereas in HEK-293 cell line, there was downregulation of *TNF- α* at all the time points tested (Fig. S(a)). In addition, other proinflammatory cytokine genes such as *IL-1 β* and *IL-6* also showed downregulation post LAT9 infection in HEK-293 cell line (Fig. S(c) and S(e)). Similar expression pattern of *TNF- α* and *IL-1 β* was observed in HeLa cell line post SO1 infection at all the time points (Fig. S(b) and S(d)). Furthermore, LAT9 infected HeLa cell line showed similar expression pattern of *TNF- α* and *IL-1 β* genes at all the time points (Fig. S(a) and S(c)) (Table 3).

Likewise, all the target cytokine genes like *TNF- α* , *IL-1 β* , *IL-6*, *IL-10* and *TGF- β* were suppressed in both HEK-293 and HeLa cell lines at 6th hpi of SO1 (Fig. (b), S(d), S(f), S(h) and S(j)) and also at 6th hpi of LAT9 in HEK-293 cell line (Fig. S(a), S(c), S(e), S(g) and S(i)) (Table 3).

On the other hand, the expression of *TGF- β* was found to be downregulated in HeLa cell line infected with SO1 (Fig. S(j)) and LAT9 strains (Fig. S(i)) at all the time points tested. However, *TGF- β* had expressed in HEK-293 cell line at 2 hpi of SO1 (Fig. S(j)) and also at 2 and 4 hpi of LAT9 (Fig. S(i)). Downregulation of *TGF- β* was observed at 6th hpi of LAT9 and SO1 on HEK-293 and HeLa cell lines (Fig. S(i) and S(j)) (Table 3).

Another anti-inflammatory cytokine, *IL-10* was downregulated in HeLa and HEK-293 cell lines when analysed at 2nd hpi and 6th hpi of SO1 (Fig. S(h) and LAT9 (Fig. S(g)) (Table 3). However, the expression level of *IL-10* for SO1 and LAT9 showed a similar pattern when analysed at similar time intervals in HEK-293 as well as in HeLa cell lines.

The pattern of *IL-6* expression varied unlike other cytokine genes targeted in the study. In HeLa cell line, the IL-6 was expressed during initial period (2nd and 4th hpi) of SO1 but it was repressed when analysed at 6th hpi (Fig. S(f)). In HEK-293 cell line, the IL-6 showed variation in the expression pattern post SO1 infection (Fig. S(f)). Moreover, the LAT9 suppressed the expression of IL-6 at all the time points on HEK-293 as well as on HeLa cell lines (Fig. S(e)) (Table 3).

Quantifying the expression of invasion genes post bacterial infection by real-time PCR

The relative mRNA expression of the SPI-1 (*invA*, *sipA* and *sipC*) and SPI-2 (*ssrB*, *ssaB* and *sseC*) genes compared to the housekeeping gene, *icdA* was determined at 6 hpi of SO1 and LAT9 in two different epithelial cell lines (HEK-293 and HeLa) which was compared with the control (bacteria infected to cell lines at zero hour). Each primer was standardized with a single peak in the melt curve and optimized at 100 nM concentration, before carrying out the expression studies using real time PCR. In HEK-293 cell line, the expression of SPI-I and SPI-II genes used in this study has shown upregulation except for *sseC* when analyzed at 6 hpi of both SO1 and LAT9. However, at similar condition, these genes showed variation in the expression pattern in HeLa cell line infected with SO1 and LAT9 strains. In HeLa cell line, the expression of *sseC* gene was downregulated post infection of SO1 and LAT9 which is comparable to HEK-293 cell infected bacterial strains. The invasion gene, *invA* showed upregulation in HeLa cell line infected with LAT9 strain whereas it downregulated in SO1 infected HeLa cells at 6 hpi. The genes *sipA* and *ssaB* showed downregulation in HeLa cell line infected with the SO1 and LAT9 strains but in HEK-293 cell line, all the genes were upregulated (*invA*: 5.976-fold more by SO1 than LAT9, *sipA*: 1.572-fold more by SO1 than LAT9, *sipC*: 2.604-fold more by LAT9 than SO1, *ssaB*: 0.446-fold more by LAT9 than SO1 and *ssrB*: 1.06-fold more by LAT9 than SO1) except for *sseC* in similar conditions. In HeLa cell line, the *sipC* gene showed upregulation when infected with SO1 whereas, downregulation was observed after LAT9 infection. Overall, except *invA* gene, the expression of *sipA*, *sipC*, *ssaB*, *ssrB* and *sseC* was downregulated at 6 hpi of LAT9 strain in HeLa cell line whereas all the above virulence genes were found to be upregulated in HEK-293 cell line except *sseC*. The two genes, *sipC* and *ssrB* were upregulated after the infection of the SO1 both in HEK-293 and HeLa cell lines. Furthermore, *sipA* and *ssaB* genes showed downregulation in HeLa cell line post infection of SO1 and LAT9 strains unlike the expression pattern of these 2 genes in HEK-293 cell line where in upregulation was observed (Fig. 6a and 6b).

Discussion

The major constraint in the cancer therapeutics is the inability of the therapeutic agents to distinguish the cancer cells from the normal cells. Therefore, contemporary methods are exploited on developing anti-cancer agents that target only cancer cells by sparing normal cells to avail progress in the cancer treatment. The non-typhoidal serovar of *Salmonella* was explored in this study for its tumor targeting potential *in vitro* via the expression of certain proinflammatory cytokine genes in epithelial cell lines. In the recent years, the non-typhoidal *Salmonella* serovars were utilized in cancer therapeutics due to its unique potential to target and accumulate inside the small and large metastatic tumors (Hoffman 2016). Several studies have carried out to check the potential of *Salmonella* Typhimurium (VP20009) in eradicating the tumor after modifying it by genetic engineering technology (Arrach et al. 2010; Hiroshima et al. 2013; Zheng et al. 2017). However, this study is the first of its kind wherein the ability of laboratory generated auxotrophic mutant NTS strain of *Salmonella* Oslo (LAT9) was checked for the production of cytokine genes in cell line model post bacterial infection.

The mutant strain (LAT9) exhibited change in phenotypic characters such as variation in morphology, growth curve, biofilm formation etc. Previous findings such as the study of Gwee et al. 2019 showed that growth defect was observed in an auxotrophic mutant when grown in minimal media, but the growth was restored when amino acids such as leucine and arginine was supplied (Gwee et al. 2019). Similarly, in the present study, the LAT9 strain did not show any growth when it was streaked on minimal media but slow and slight growth was observed when the growth media was supplemented with amino acids leucine and arginine which is also in accordance with the study of Lv *et al.* 2015 wherein they showed the growth of mutant phenotypes of *Natrinema* species which was auxotrophic to leucine, lysine and arginine grew on minimal medium with the nutritional supplements (Lv et al. 2015). In addition, growth defect of LAT9 in the minimal media was cured when the bacteria was grown on a complete media. Therefore, LAT9 was further examined for other phenotypic characteristics. Morphologically, the LAT9 appeared small in size (approximately 1:10) when compared with SO1 after plating the strain on a non-selective agar media (Fig. 2) which was comparable with the study of Lv et al. 2015 wherein the amino acid auxotrophs of haloarchean generated showed small sized colonies on minimal media supplied with the relevant amino acids (Kwan et al. 2015).

In terms of growth rate, the mutant showed significant reduction in the initial lag phase as well as in the overall growth in comparison with the parent strain, SO1. A study using auxotrophic mutant of *Salmonella enterica* conducted by Kwan et al. 2015 found that phenotypic growth and metabolism of amino acid auxotrophs was influenced by the uniqueness of each nutrient and they concluded the necessity of production of certain amino acids by this bacterium by itself since nutrients regulate its growth (Kwan et al. 2015). They also proved that interruption of amino acid pathways decreased the load of mutant *Salmonella enterica* recovered from the spermosphere and early rhizosphere of alfalfa seedlings when compared to the wild-type bacteria (Kwan et al. 2015). In addition, a study of Gordhan *et al.* 2002 demonstrated that L-arginine auxotrophic mutant of *Mycobacterium tuberculosis* showed decline in the growth rate upto undetectable level post 14 days of inoculation in the culture from 10^6 CFU/mL when assessed in the growth medium (Middlebrook 7H9 medium) without the supplements of amino acid arginine when compared to wild-type bacteria, but it has reached to stationary phase with an optical density similar to the wild-type in the medium containing the maximum amino acid supplement although it showed a delayed lag phase (Gordhan et al. 2002). Therefore, with respect to growth kinetic study of mutant and wild-type strains, our study showed similarity to the study of Gordhan *et al.*

2002 (Gordhan et al. 2002). Previous *in vitro* study carried out using *Salmonella* Typhimurium harbouring *aroA* mutation was found to be less motile than the parent strain, however the negative staining results of electron microscopy revealed the presence of flagella in both the strains although the mutant had impaired motility (Felgner et al. 2016). Hence, the motility test carried out in the present study revealed that the strain LAT9 was comparatively less motile than SO1. Therefore, we assume that the alteration in the growth rate of LAT9 must have resulted in its lower motility. In addition to all these tests, the mutant was also subjected for its biofilm forming ability, wherein it revealed that the wild-type *Salmonella* is a strong biofilm former whereas the LAT9 strain has lost its ability to form biofilm. The alteration in the biofilm forming ability might be due to the mutation in the genes that affected its growth rate, motility and caused other phenotypic defects (Chorobik et al. 2013). However, this needs further investigation in our study.

Since, *Salmonella* is an intra-cellular pathogen and its invasion is a dynamic phase to produce the oncolytic activity (Kim et al. 2015), we evaluated the invasion and replication potential of the mutant strain. The mutant strain showed significantly higher invasiveness (1.67-fold > by LAT9 than SO1) than wild-type strain *in vitro* in the epithelial cell lines tested while its replication was significantly reduced than the SO1 (Fig.5). Similar results were observed in a study conducted by Zhao *et al.* 2006 wherein they showed that *S. Typhimurium* A1-R mutant Leu-Arg auxotroph could efficiently invade HT-29 tumour cells (Zhao et al. 2006). Moreover, the lower replication rate may be due to its slower growth rate which is in comparable to the study of Felgner et al. 2016 wherein they found that *aroA* deletion mutant exhibited lower replication in J774 cell line (macrophage like cell line) whereas replication of the wild-type strain was more (Felgner et al. 2016).

In order to determine the cytotoxicity of the auxotrophic mutant in the cell line model system, we have administered bacterial strains of wild-type and mutant to HeLa and HEK-293 cell lines at an m.o.i. of 10 and monitored the cytotoxic potential of the bacterial strains from 0-24 h by performing LDH assay for all the time points of infection at an interval of an hour. Cent percent cytotoxicity was attained at 18th hour post LAT9 and SO1 infection in HEK-293 cell line whereas in HeLa cell line complete cell death was observed in between 22nd and 24th hour of bacterial infection. Thus, from these results we assumed that cytotoxicity of the bacterial strains varied in cell lines at different time points. Although, there was a variation in the trend of cell death at different time intervals in these two cell lines, the auxotrophic mutant retained the cytotoxic potential similar to the wild-type strain. A previous study demonstrated the histological analysis for cytotoxic potential of the *Salmonella* Typhimurium in inducing necrosis of tumor and causing apoptosis wherein they hypothesised that these mechanisms might be as result of the triggering of TNF-alpha by *Salmonella* Typhimurium (Leschner et al. 2009). Consequently, the damage caused to the normal cell by the *Salmonella* Typhimurium would be less because of its ability to lyse the tumor cells from within (Westphal et al. 2008). Therefore, it suggests that bacteria club together mostly in the immunity confined region and not in the external layer of tumor tissue where tumor cells multiply (Chen et al. 2015). Hence, in this study we assume that auxotrophic mutant (LAT9) might selectively target tumor cells in the higher animal model system since it causes lysis of tumor cells.

As the expression of proinflammatory cytokines plays a very important role in tumor regression, both LAT9 and SO1 strains were checked for its ability to trigger cytokine response by determining the expression of cytokine genes in HEK-293 and HeLa cell lines at different time intervals after exposure. Tumor Necrosis Factor-alpha (TNF- α) plays as a key arbitrator in bacteria mediated tumor therapy. Upon systemic infection of the bacteria, necrosis is triggered by TNF- α in tumor tissues (Felgner et al. 2018;Jia et al. 2020). At the time of tumor lysis,

cytokines such as Interleukin-1 beta (IL-1 β) and TNF- α plays a crucial role (Phan et al. 2015; Zheng et al. 2017). In the present study, *TNF- α* and *IL-1 β* were expressed at 6 hpi of LAT9 on HeLa cells (Fig. S(a) and S(c)). This observation is similar to the study of Kim et al. 2015 wherein they proved that *Salmonella* infection lead to increased infiltration of pro-inflammatory cytokines such as IL-1 β and TNF- α that lead to tumor destruction. Apoptosis was induced by TNF- α and T-cells (involved in innate and adaptive immune system) that lead to tumor cell lysis (Kim et al. 2015). Felgner et al. 2018 also revealed the elevated levels of TNF- α in the tumors infected with SF200 (Felgner et al. 2018). Similar to earlier studies, this study also showed the production of cytokine genes such as *TNF- α* and *IL-1 β* that leads to the death of cancer cell lines at 6 hpi of LAT9.

In addition, Interleukin-10 (IL-10) and Tumor Growth Factor-beta (TGF- β) were downregulated in HeLa cell line when analysed at 6 hpi of LAT9 (Fig. S(g) and S(i)), this data is in agreement with a study carried out using *Listeria monocytogenes* which was analysed for its tumor inhibiting ability wherein they showed the reduction in the cytokines such as IL-10 and TGF- β and the activation of IL-2, IL-6, IL-12, and TNF- α during immunostimulation in tumor microenvironment (Masjedi et al. 2018, Ebbing et al. 2019). TGF- β is known to orchestrate the cancer cell progressions during the late stage by the induction of mesenchymal phenotype in the epithelial tumors, a process where epithelial to mesenchymal transition (EMT) occurs (Löffek 2018). Furthermore, Löffek 2018, revealed that tumor patients had advantageous effect when TGF- β was blockaded and hence, it caused offset of tumor relapses (Löffek 2018). Consequently, the preclinical studies carried out in mouse models found the reduction in the advancement of recognised osteolytic lesions and also the reduced progression of melanoma bone metastasis after targeting the TGF- β therapeutically (Mohammad et al. 2011). Tumor growth was decreased in B16F0 melanomas and augmentation of the antitumor immunity was observed in C57BL/6 mice when the TGF- β was silenced (Tai and Wang 2013). The plasma levels of TGF- β was increased in patients with renal cell carcinoma, colon cancer, breast cancer and melanoma which resulted in poor clinical outcome besides the development of metastasis and the advancement of tumor. Therefore, many *in vitro* and *in vivo* studies demonstrated the clinical stand point for the hindrance of TGF- β signalling in cancer treatment (Wikström et al 1998; Junker et al. 1996; Shariat et al. 2002; Reiss and Barcellos-Hoff 1997). Thus, a decrease or downregulation of TGF- β on LAT9 exposure in our study shows that the mutant strain (LAT9) might replicate the same results in higher animal models and could become a candidate strain to be used for anticancer therapy in the future.

The role of IL-10 was associated with the production of immune suppressive tumor microenvironment (O'Garra and Vieira 2007). Interleukin-10 also hinders the secretion of proinflammatory cytokines like TNF- α , IL-1 β , IL-6, IFN-gamma etc. by CD4⁺ T-cells *in vitro* (Moore et al. 2001; Mumm et al. 2011). Therefore, in this study we have observed the reduced expression of IL-10 and increased expression of TNF- α at 6 hpi on HeLa cell line. This might result in tumor cell regression in higher animal models.

Interleukin-6 (IL-6) is a pro-inflammatory cytokine produced by different cell types such as helper T (Th) cells, tumor associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and fibroblasts in the tumor microenvironment including tumor cells which plays a crucial role in progression and differentiation of cancer cells (Moore et al. 2001; Bromberg and Wang 2009; Phan et al. 2015). In several types of cancer, elevated levels of IL-6 has been validated inside the tumor site and also in serum which thereby resulted in poor prognosis and reduced life span of the patients whereas better treatment response was achieved as a result of decline in the IL-6 level (Grivennikov S, Karin M. 2008; Guo et al. 2012; Dethlefsen et al. 2013; Shibayama et al. 2014) The

characteristic features of the tumor cell such as apoptosis, proliferation, metabolism, angiogenesis, survival, and metastasis can be exaggerated by IL-6 (Kumari et al. 2016). Resistance caused to tumor therapy such as multi drug resistance is also modulated by IL-6 (Ghandadi and Sahebkar 2006). Study carried out using LAT9 showed down regulation of IL-6 at all the time points except at 4 hpi in HeLa cell line (Fig. S(e)) which is in contrast to many previous studies wherein they found the high concentration of IL-6 in the tumor microenvironment and its association in cancer progression. These results showed that LAT9 inhibited the propagation of HeLa cell line by the recruitment of certain proinflammatory cytokines and by inhibiting tumor promoting cytokine genes in the tumor cells at specific time point there by displaying the features of a potential agent that might inhibit the growth of tumor cells in higher animal models.

Meanwhile, all the above cytokine genes were downregulated in HEK-293 cell line at 6 hpi of LAT9. The expression of the above cytokine genes were also analysed at 3 more different time points *viz* 2 h and 4 h post LAT9 infection to HeLa and HEK-293 cell lines. At all these time points, TGF- β showed downregulation (Fig. S(i)) whereas TNF- α and IL-1 β showed upregulation from 4 h post LAT9 infection on HeLa cell line (Fig. S(a) and S(c)). Similar experiments were carried out using SO1 on HEK-293 and HeLa cell lines in order to compare the difference in the expression of cytokine genes. Contrasting results were obtained at 6 hpi of SO1 on HEK-293 and HeLa cell lines when compared to LAT9 infection. All the cytokine genes were downregulated in both the cell lines at 6 hpi of SO1 and the results were extreme but it is to some extent comparable to the expression shown at 2 hpi of LAT9 on HEK-293 and HeLa cell lines except for TGF- β where in it was upregulated in HEK-293 cell line at 2 hpi of LAT9. However, the expression of TGF- β post SO1 infection in HeLa cell line showed similar trend to that obtained as a result of LAT9 infection wherein it was downregulated at all the time points tested (Fig. S(i) and S(j)). Hence, the results obtained in this study suggests that at different time of bacterial infection onto HeLa and HEK-293 cell lines, the expression of cytokine genes varied considerably in either of the cell lines except for few cytokine genes. Encouraging results were obtained at 6th hpi of LAT9 on HeLa cell line. Therefore, the virulence nature of the LAT9 at 6 hpi was analysed by performing expression studies targeting SPI-1 and SPI-2 genes and also compared it with the virulence of SO1 on both HEK-293 and HeLa cell lines.

In order to find the virulence nature of the LAT9 strain when compared to SO1, virulence genes (*invA*, *sipA*, *sipC*, *ssaB*, *ssrB* and *sseC*) of *Salmonella* Pathogenicity Islands one and two (SPI-1 and SPI-2) which aid in bacterial cell invasion and intracellular replication were selected to check the expression level in the epithelial cell lines, HEK-293 and HeLa respectively. All the virulence genes tested showed upregulation at 6 hpi of SO1 and LAT9 strains in HEK-293 cell line except *sseC* gene (Fig. 6b), which showed down regulation in both the strains. However, variable results were seen in HeLa cell line infected with LAT9 and SO1 (Fig. 6a). At 6 hpi of LAT9 on HeLa cell lines, all the virulence genes showed downregulation except *invA* whereas only *sipC* and *ssrB* showed its expression after SO1 infection. When the virulence genes expression was compared between the epithelial cell lines after the infection of LAT9, all the genes were found to be downregulated except *invA* gene in HeLa cell line whereas all the genes were upregulated except *sseC* in HEK-293 cell line. Likewise, SO1 also showed upregulation of all the virulence genes except *sseC* in HEK-293 cell line (Fig. 6b). Thus, at similar condition both wild-type and the mutant strain expressed the virulence genes in similar fashion in HEK-293 cell line but its expression in HeLa cell lines varied between the bacterial strains considerably. This is in agreement with the study of Gwee et al. (2019) wherein they showed that though the growth of auxotrophic mutant of the *S. Agona* was defective, the invasiveness of the strain was unaffected *in vitro* (Gwee et al. 2019).

From the above results, we can infer that at 6 hpi of LAT9 on HeLa cell line showed less virulence compared to SO1 but retained its invasion potential and it is comparable with the results obtained for epithelial cell invasion of LAT9 (Fig. 5a and 5b) wherein it showed higher invasion than SO1. This signifies that the mutant NTS (LAT9) possess the ability to trigger cytokine gene expression thereby gained immunogenicity along with simultaneous reduction of its virulence property. Therefore, from this study we have observed that LAT9 has the favourable positive attributes of a therapeutic agent which is responsible to eradicate the expansion of cancer cells. However, protein level validation of the cytokines, mechanism of regulation of cytokines and *in vivo* studies are necessary to confirm the antitumor properties of LAT9.

Conclusion

The study sheds light on the positive attributes of non-typhoidal *Salmonella* serovar to be used as an anticancer agent in higher animal models in future studies. The auxotrophic mutant characterised in the study triggered the expression of proinflammatory cytokine genes such as *TNF- α* and *IL-1 β* in cell line model that otherwise plays a very important role in tumor regression. Furthermore, the mutant strain showed lower virulence, but it retained higher invasion potential which is regarded as the positive feature of an ideal tumoricidal bacterial agent. To the best of our knowledge, this is the first study carried out using a less pathogenic strain of NTS, *Salmonella* Oslo in order to explore its positive effects in combating the cancer cells effectively *via* the activation of certain cytokine genes and thus, aiming to replicate similar results in animal models. Though a preliminary study, it tried to unravel the potential of a less pathogenic microbe which can be utilized in clinical application in a near future in order to combat tumors. Hence, the finding of this study is an important step towards the clinical application. Nevertheless, the application of the attenuated *Salmonella* Oslo generated in the present study needs further investigation in vivo animal models to be successfully used as an anticancer agent.

Declarations

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Conflict of interest

The authors have no financial conflict of interest to declare

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Tables

Table 1 Primer details of cytokine genes

Target Genes	Primer sequences (5'- 3')	Annealing temperature (°C)	Product size (bp)	References
<i>TNF-α</i>	F: CCCAGGGACCTCTCTCTAATC R: ATGGGCTACAGGCTTGTCCT	55	84	(Stordeur et al. 2002)
<i>IL-1β</i>	F: ACAGATGAAGTGCTCCTTCCA R: GTCGGAGATTCTAGCTGGAT	55	73	(Giribaldi et al. 2010)
<i>IL-6</i>	F: GGTACATCCTCGACGGCATCT R: GTGCCTCTTTGCTGCTTTCAC	55	61	(Xu et al., 2012)
<i>IL-10</i>	F: CATCGATTTCTTCCCTGTGAA R: TCTTGGAGCTTATTAAAGGCATTC	55	74	(Stordeur et al. 2002)
<i>TGF-β</i>	F: GCCCTGGACACCAACTATTGCT R: AGGCTCCAAATGTAGGGGCAGG	55	161	(Jung et al. 1995)
<i>RP II</i>	F: CTTACGGTGCTGGGCATT R: GTGCGGCTGCTTCCATAA	55	239	(Mehta et al. 2010)

Table 2 Primer details of virulence genes

Target Gene	Primer sequences (5'- 3')	Annealing temperature (°C)	Product size (bp)	References
<i>invA</i>	F:GTGAAATTATCGCCACGTTCTGGGCAA R: TCATCGCACCGTCAAAGGAACC	54	284	(Rahn et al. 1992)
<i>sipA</i>	F: CCATTGACTAACAGCAGCA R: CGTGACCACCTTTCCATCTT	54	199	This study
<i>sipC</i>	F: GTACCGATGCGACGAAAAAT R: ATCGATTCTGGGTCATATCCA	54	197	This study
<i>ssaB</i>	F: GGATTCATGCTGGCAGTTTT R: TGCTGCAAGCAGTAGTGTC	54	207	This study
<i>ssrB</i>	F: AATGCCTGTTGTGCATACGA R: TTAGCACCTGCGGCTAAAGT	54	176	This study
<i>sseC</i>	F: CAGATTGGCCGTGCTTTTAT R: ATTCGCCATAGCCATTTTAC	54	210	This study
<i>icdA</i>	F:TGGTATCGGTGTTGATGTCACTC R:CATCCTGGCCGTAAACCTGTGTG	54	140	(Parsons and Heflich 1997)

Table 3 Relative expression levels of cytokine genes post bacterial invasion on HEK-293 and HeLa cell lines

Time points	Cytokine genes	Relative gene expression				Relative gene expression		Ratio	<i>p</i> -value
		LAT9 on HEK-293	LAT9 on HeLa	Ratio	<i>p</i> -value	S01 on HEK-293	S01 on HeLa		
2h	<i>TNF-α</i>	0.069 ± 0.0519	0.0916 ± 0.0200	0.75	>0.05	27.1979 ± 0.9329	72.949 ± 7.4952	0.3728	<0.05
	<i>TGF-β</i>	2.4005 ± 1.3949	0.6784 ± 0.0199	3.5384	>0.05	12.7857 ± 0.8141	0.1363 ± 0.0678	93.805	<0.05
	<i>IL-1β</i>	0.0026 ± 0.0028	0.4035 ± 0.1413	0.0064	<0.05	0.8184 ± 0.2372	4.7372 ± 1.0817	0.1727	<0.05
	<i>IL-6</i>	0.0164 ± 0.0197	0.1373 ± 0.0326	0.1194	>0.05	0.162 ± 0.0961	5.0587 ± 2.5593	0.032	<0.05
	<i>IL-10</i>	0.1754 ± 0.2056	0.0141 ± 0.0061	12.5	>0.05	0.3072 ± 0.1810	0.2504 ± 0.2044	1.2268	>0.05
4h	<i>TNF-α</i>	0.0492 ± 0.0238	1.7254 ± 0.1267	0.0285	<0.05	14.4427 ± 2.0459	532.4358 ± 33.9021	0.0271	<0.05
	<i>TGF-β</i>	1.9747 ± 1.3642	0.7149 ± 0.2372	2.7622	>0.05	0.0262 ± 0.0158	0.2932 ± 0.2225	0.0893	>0.05
	<i>IL-1β</i>	0.0168 ± 0.0151	4.2247 ± 0.6599	0.0039	<0.05	0.3198 ± 0.014	39.1570 ± 6.8735	0.0081	<0.05
	<i>IL-6</i>	0.0741 ± 0.0489	1.0081 ± 0.4664	0.0735	<0.05	1.6297 ± 1.6674	4.1833 ± 2.5988	0.3895	>0.05
	<i>IL-10</i>	0.9334 ± 0.7296	8.0848 ± 0.1981	0.1154	<0.05	0.342 ± 0.0748	27.2083 ± 16.4697	0.0125	<0.05
6h	<i>TNF-α</i>	0.4577 ± 0.0847	10.1883 ± 1.5914	0.0449	<0.05	0.3983 ± 0.0564	0.6885 ± 0.6507	0.5785	>0.05
	<i>TGF-β</i>	0.0012 ± 0	0.4375 ± 0.1082	0.0027	<0.05	0.0561 ± 0.0235	0.0263 ± 0.0239	2.133	>0.05
	<i>IL-1β</i>	0.0497 ± 0.018	1.5217 ± 0.0671	0.0326	<0.05	0.1239 ± 0.0695	0.0716 ± 0.0962	1.7304	>0.05
	<i>IL-6</i>	0.289	0.9328	0.3098	<0.05	0.0751	0.0698 ±	1.0759	>0.05

	$\pm$ 0.0325	$\pm$ 0.4957			$\pm$ 0.0277	0.0946		
<i>IL-10</i>	0.1739 $\pm$ 0.011	0.1602 $\pm$ 0.1124	1.0855	>0.05	0.0344 $\pm$ 0.0325	0.0288 $\pm$ 0.0089	1.1944	>0.05
Ratio: Bacterial invasion on HEK-293 to HeLa cell lines								

Figures

Figure 1

Representative images of the *leuB/argH* transformant of nontyphoidal Salmonella (NTS) obtained after electroporation of PCR-products of *leuB/argH* Kan cassette into electrocompetent lambda Red plasmid. Plates A, B and C represents the difference in the growth pattern shown by auxotrophic mutant (LAT9) and wild-type (SO1) strains when streaked on minimal media without any amino acid supplements (a), minimal media supplemented with amino acids leucine and arginine (b) and on complete media (c).

Figure 2

Appearance of wild-type (SO1) (a) and auxotrophic mutant (LAT9) (b) on LB agar media.

Figure 3

Auxotrophic mutant (LAT9) and wild-type (SO1) nontyphoidal *Salmonella* stabbed into soft agar and incubated for 4 hpi (a) and 24 hpi (b) respectively.

Figure 4

Growth kinetics of SO1 and LAT9 in non-selective broth media. Growth rate of LAT9 was slower with more than 4 h of lag phase when compared with the SO1 which had a lag phase of 1.5-2 h. Gradual rise in the optical density was observed after 4 h by the LAT9 whereas SO1 exhibited rapid increase in optical density from 2nd h onwards. Two-way ANOVA was performed to analyse the significant difference between the growth of the strains. The asterisks indicate a significantly different optical density of two bacterial strains at various time points (** $p < 0.001$). Results are replicative of atleast 3 independent experiments.

Figure 5

Epithelial cell invasion of SO1 and LAT9 at 2 hpi (a and b); Intracellular replication and survival of SO1 and LAT9 at 16 hpi (c and d) in HeLa and HEK-293 cell lines respectively. Unpaired t-test was used to find out the statistical significance between the strains. Data represent the mean ± SD. The asterisks indicate a significantly different bacterial strains during epithelial cell invasion and intracellular replication in HeLa and HEK-293 cell lines (***)p < 0.001). Results are replicative of atleast 3 independent experiments

Figure 6

Graphical representation of the virulence gene expression on HeLa (a) and HEK-293 (b) cell lines at 6 hpi of LAT9 and SO1. One-way ANOVA was used to check the statistical significance between the strains. Data represent the mean $\pm$ SD. Results are replicative of atleast 3 independent experiments

Supplementary Files

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