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Inducing meningococcal meningitis serogroup C in mice via intracisternal delivery

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DIPARTIMENTO DI MEDICINA MOLECOLARE E BIOTECNOLOGIE MEDICHE

Naples, July 19, 2019

Dr. Vineeta Bajaj
Review Editor
JoVE

Dear Editor,

In reply to your letter of 12 July 2019, we submit a revised version of the manuscript JoVE60047R2, entitled "Inducing meningococcal meningitis serogroup C in mice via intracisternal delivery" addressing all comments raised by the Editor.

Please find below:

1. the revised manuscript;
2. the revised manuscript with marked (green highlighted) changes;
3. the accompanying letter, referring to the marked version of the manuscript that describes how we have met each of the Editor' suggestions and how the manuscript has been modified.

All the authors are aware of and agree to the content of the paper and their being listed as authors on the paper.

Thank you in advance for your consideration,

Best regards

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TITLE:

Inducing Meningococcal Meningitis Serogroup C in Mice via Intracisternal Delivery

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KEYWORDS:

Infection; *Neisseria meningitidis*; meningococcal meningitis; mouse models; intra-cisternal injection; brain tissue; isogenic mutant strain.

SUMMARY:

Here, we describe a method to induce meningococcal meningitis through an intracisternal route of infection in adult mice. We present a step by step protocol of meningococcal infection from the preparation of inoculum to the intracisternal infection; then record the animal survival and evaluate the bacterial loads in murine tissues.

ABSTRACT:

Neisseria meningitidis (meningococcus) is a narrow-host-range microorganism, globally recognized as the leading cause of bacterial meningitis. Meningococcus is a transient colonizer of human nasopharynx of approximately 10% of healthy subject. In particular circumstances, it acquires an invasive ability to penetrate the mucosal barrier and invades the bloodstream causing septicaemia. In the latest case, fulminating sepsis could arise even without the consequent development of meningitis. Conversely, bacteria could poorly multiply in the bloodstream, cross the blood brain barrier, reach the central nervous system, leading to fulminant meningitis. The murine models of bacterial meningitis represent a useful tool to investigate the host-pathogen

interactions and to analyze the pathogenetic mechanisms responsible for this lethal disease. Although, several experimental model systems have been evaluated over the last decades, none of these were able to reproduce the characteristic pathological events of meningococcal disease. In this experimental protocol, we describe a detailed procedure for the induction of meningococcal meningitis in a mouse model based on the intracisternal inoculation of bacteria. The peculiar signs of human meningitis were recorded in the murine host through the assessment of clinical parameters (e.g., temperature, body weight), evaluation of survival rate, microbiological analysis and histological examination of brain injury. When using intracisternal (i.cist.) inoculum, meningococci complete delivery directly into *cisterna magna,* leading to a very efficient meningococcal replication in the brain tissue. A 1,000-fold increase of viable count of bacteria is observed in about 18 h. Moreover, meningococci are also found in the spleen, and liver of infected mice, suggesting that the liver may represent a target organ for meningococcal replication.

INTRODUCTION:

Neisseria meningitidis is a Gram negative β-proteobacterium restricted to the human host, well known for being one of the most common causes of meningitis and sepsis in the human population across the world. It colonizes the upper respiratory tract (nose and throat) of healthy and asymptomatic carriers (2-30% of the population), but the bacterium sometimes evades various host immune defenses and spreads from the bloodstream to the brain causing an uncontrolled local inflammation, known as meningococcal meningitis. A combination of host and bacterial factors appears to contribute to the transition from the commensal to the invasive behavior[1].

N. meningitidis is specialized exclusively in human colonization and infection. It has a narrow host range and, therefore, has limited in vivo pathogenesis studies due to the lack of suitable animal models that reproduce the human meningococcal disease. As a result, it had led to fundamental gaps in the comprehension concerning the pathogenesis of septicemia and meningitis caused by meningococcus. In the last decades, the development of many in vitro systems allowed the identification of several meningococcal virulence factors[2-4]. Although these valuable studies provided important insights to understand the role of these factors for a successful meningococcal infection, these models did not allow assessment of the consequences of bacterial interactions with the humoral and cellular immune system and even less with the whole tissue. In vivo animal models of infection are of great relevance as well for the evaluation of protection degree conferred by vaccine formulations. As a human-tropic pathogen, meningococcus possess appropriate determinants necessary for successful infection such as surface structures (i.e., type IV pili and opacity proteins) and iron uptake systems for human receptors and transport proteins (i.e., transferrin and lactoferrin)[5-7] to properly adhere, survive and invade the human host. Finally, the genetic variation abilities of the pathogen to evade and/or block the human immune response further contribute to the high species tropism[8-9]. Therefore, the absence of specific host factors, involved in the interaction, can block steps of the pathogen's life cycle, establishing significant difficulties in the development of small animal models summarizing the meningococcal life cycle.

Over the past decades, several approaches have been developed to improve our understanding of the meningococcal infectious cycle. Infections of two animal model, mouse and rat, either intraperitoneally (i.p.) or intranasally (i.n.), were developed to reproduce meningococcal disease[10-17]. The laboratory mouse is probably one of the more versatile animals for inducing experimental meningococcal infection.

However, the i.p. way of infection leads to the development of severe sepsis although it does not mimic the natural route of infection, whereas the i.n. route of infection was useful to evaluate meningococcal pathogenesis, even though it may induce lung infection prior to sepsis[10-17].

The i.p. mouse model was instrumental to assess the protection from the meningococcal challenge[10-12]. The mouse model of meningococcal colonization based on the i.n. route of infection has been developed with infant mice, as they are more susceptible to meningococci, to reproduce an invasive infection mimicking the course of the meningococcal disease in humans[13-17]. Moreover, to promote meningococcal replication in the murine host, a growing number of technical strategies were also applied including the administration of the iron to the animals to improve the infection, the use of high bacterial *inoculum*, mouse-passaged bacterial strain as well as the employment of infant or immunocompromised animal hosts[10,13,15,18,19]. Expression of specific human factors like CD46[20] or transferrin[21] has increased the susceptibility of mice to this human-tropic bacterium; the employment of the human skin xenograft model of infection has also been useful to evaluate the adhesion ability of meningococci to human endothelium[22-23]. Collectively, the recent development of humanized transgenic mice has improved the understanding of the meningococcal pathogenesis and its host interactions.

Previously, we developed a murine model of meningococcal meningitis where the inoculation of bacteria was performed into the *cisterna magna* of adult mice with mouse-passaged bacteria[24]. Clinical parameters and the survival rate of infected mice demonstrated the establishment of meningitis with characteristics comparable to those seen in the human host, as well as, the microbiological and histological analyses of the brain. From these infected mice, bacteria were, also, recovered from blood, liver, and spleen, and bacterial loads from peripheral organs correlated with the infectious dose. In particular, this model was employed to evaluate the virulence of an isogenic mutant strain defective in the L-glutamate transporter GltT[24]. Recently, using our mouse model of meningococcal meningitis based on i.cist. route with serogroup C strain 93/4286[2,24] and an isogenic mutant defective in *cssA* gene encoding for UDP-N-acetylglucosamine 2-epimerase[25], we have analyzed the role of exposed sialic acid in the establishment of disease in mice.

In this protocol, we describe a straightforward method to induce experimental meningococcal meningitis based on the i.cist. route of infection in Balb/c adult mice. This method is particularly useful for the characterization of meningococcal infection in a murine host, as well as for the assessment of the virulence between wild type reference strains and isogenic mutants. The intra-cisternal route of infection ensures complete delivery of the meningococci directly into the *cisterna magna*, which in turn facilitates bacterial replication in the cerebrospinal fluid (CSF) and induces meningitis with features that mimic those present in humans[2,24-26].

PROTOCOL:

This protocol was conducted to minimize animal suffering and reduce the number of mice in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). In vivo experiments reported in this study were approved by the Ethical Animal Care and Use Committee (Prot. number 2, 14 December 2012) and the Italian Ministry of Health (Prot. number 0000094-A-03/01/2013). All the procedures should be performed inside the Biosafety Cabinet 2 (BSC2) in a BSL2 room, and the potential infected waste should be disposed in dedicated containers.

1. Infection of mice with *N. meningitidis* Serogroup C strain

CAUTION: *N. meningitidis* is potentially a harmful pathogen and all necessary precautions must be taken when handling this microorganism. The entire experimentation requires Biosafety Level 2 (BSL2) containment. The researcher involved in animal studies should wear disposable personal protective equipment (PPE) for the duration of the experiment.

1.1. Preparation of bacteria for in vivo infection studies

1.1.1. Pick a single colony from a fresh *Neisseria meningitidis* culture on GC (Gonococcal) agar plate supplemented with 1% (vol/vol) Polyvitox supplement and inoculate in 10 mL of GC broth.

1.1.2. Grow the bacteria at 37 °C in an orbital shaker incubator with the speed of 220 rpm. Keep checking the O.D. of the culture with a spectrophotometer. Grow the culture until the early exponential phase at an optical density OD_{600nm} of 0.7, corresponding to ≈ 7 x 10^8 CFU/mL.

1.1.3. Once the required O.D. is obtained, make frozen stocks by adding 10% glycerol. Dispense 1 mL of the culture in the cryovials. Store the vials at -80 °C until use.

NOTE: Although it is better to use fresh bacterial culture, frozen stocks were used to simplify and standardize the in vivo experiment. Usually the frozen stocks have been employed within approximately 6 months from the preparation.

1.1.4. Before the infection, thaw frozen bacteria at room temperature.

1.1.5. Harvest the bacterial cells by centrifugation for 15 min at 1,500 x *g* and resuspend in 1 mL of fresh GC broth containing iron dextran (5 mg/kg).

NOTE: The GC broth is prepared with the addition of iron dextran (5 mg/kg) in order to favor the replication of meningococci in the host tissue[14,18,27].

1.1.6. Before using, perform viable counts of bacteria to determine the exact number of CFU for infection. To do so, pick up 10 µL of bacterial suspension and proceed with serial dilutions and

spread each dilution on GC agar plates and incubate at 37 °C with 5% CO_2, for 18-24 h.

1.2. Intra-cisternal injection of mice

NOTE: The entire procedure is performed in the laminar flow cabinet to maintain aseptic conditions.

1.2.1. House laboratory mice (eight-week-old, female Balb/c) under specific pathogen free conditions. Provide food pellets and water *ad libitum*.

1.2.2. Settle the animals in the new environment for 1 week before starting the experiment.

1.2.3. Before starting the experiment, weigh and evaluate their body temperature.

NOTE: The inbred Balb/c female mice of eight-week-old weigh an average of about 19 g. The average temperature of laboratory mice physiologically ranging from 36-38 °C[24].

1.2.4. Scruff the animal from the neck, proceed with the 70% ethanol disinfection of the abdomen and inject i.p. iron dextran (dissolved in 1 % phosphate saline buffer, 250 mg/kg) in the lower right quadrant of the murine abdomen by using a 25 G needle 0.5 mm x 16 mm, approximately 2-3 h before the infection.

NOTE: The intraperitoneal injection was performed in the lower right quadrant of the murine abdomen to avoid damaging abdominal organs such as urinary bladder, cecum, etc. The administration of exogenous iron source, in the form of iron dextran, to animals prior to the infection favors the bacterial multiplication in the host[14,18,27].

1.2.5. After 2-3 h, perform animal anesthesia with ketamine (50 mg/kg) and xylazine (3 mg/kg).

1.2.6. Check for the depth of anesthesia by ensuring the absence of pain response upon pinching the toe.

1.2.7. Position the mouse in sternal decubitus and carefully stretch the limbs and the cervical spine to keep the vertebral column in a straight position.

1.2.8. Gently mix the bacterial suspension to maintain a consistent suspension before loading a syringe of 30 G needle x 8 mm.

NOTE: Prepare the bacterial suspension as close as possible to the injection time; meanwhile, store it at room temperature.

1.2.9. Based on the post thawing bacterial titer (CFU/mL), proceed with the calculation of the total CFU to be used, with respect to the total number of animals to be infected (CFU bacterial dose per number of animals). Proceed with the calculation of the exact volume to be taken from

the vial to obtain the total CFU applying a proportion between the post thawing bacterial titer (CFU/mL) and the total CFU useful for the infection of n mice (post thawing bacterial titer CFU/ml= total CFU: x). Establish the final volume with respect to the total number of animals to be infected.

NOTE: In these experiments, a wide range of titer from 10^4 to 10^9 per animal was used.

1.2.10. Proceed with the 70% ethanol disinfection of the surgical area (head).

1.2.11. Identify the injection point with the help of a needle and inject the established CFU of meningococci (wild type strain and isogenic mutant strain), or GC broth supplemented with iron dextran (5 mg/kg) as control, in a total volume of 10 µL into the *cisterna magna* of mice through an occipital burr hole by using a 30 G needle x 8 mm.

1.2.11.1. Perform the cisternal inoculum by placing the needle at the craniocervical junction, specifically in the dorsal subarachnoid space. Ventroflex the head the to make this space accessible[28].

1.2.11.2. Briefly, place the animal in lateral recumbency, hold the ears out of the way and flex the neck flexed moderately (90 to 100°) at the cisternal region. Ensure that the midline of the neck and the head (from the nose to the occiput) are in perfectly parallel position to the tabletop.

1.2.11.3. Touch the atlas wings and make sure that they overlap, eliminating axial rotation. A natural indentation can usually be touched on midline where the needle is most likely to enter the occipital hole.

1.2.11.4. Discard the syringe and needle safely after the injection of mice with *Neisseria*.

1.2.12. Place the animal in the cage and wait for the awakening and full recovery of movement.

1.2.13. Keep the cages with infected mice under a laminar flow cabinet.

1.2.14. Monitor mice, 24 h post infection, for clinical signs of coma according to the coma scale[29]. Coma scale: 1 = coma, 2 = does not stand upright after being turned on the back, 3 = stands upright within 30 s, 4 = stands upright within 5 s, minimal ambulatory activities, 5 = normal.

1.2.15. Proceed for the animal survival or CFU counts assay on the infected animals as detailed in step 2.

1.2.16. Perform euthanasia of mice with a score of 2 by cervical dislocation and record as dead for statistical analysis.

2. Animal survival and CFU counts

2.1. Animal survival

2.1.1. Prepare bacterial inocula at different doses (ranging from 10^4 to 10^9 CFU per mouse) to infect animals by the i.cist. route (see step 1.2).

2.1.2. Inoculate control mice with GC broth, supplemented with iron dextran (5 mg/kg), in the same manner.

2.1.3. Monitor animals for clinical symptoms: ruffled fur, hunched appearance, hypothermia, weight loss, lethargy, or moribund[24-26,30], every day throughout the whole experiment for 168 hours (7 days).

2.1.4. Measure the body weight and temperature by using a digital balance and a rectal thermometer, respectively.

2.1.5. Record the survival of mice for a week.

NOTE: Record the natural death of animal post infection while the animals that reach a coma value of 2 or that survive over 168 h of observation will be euthanized.

2.1.6. Anesthetize mice with a coma value of 2 or that survive over the observation time with ketamine (50 mg/kg) and xylazine (3 mg/kg).

2.1.7. Check that the pain response is absent by toe pinch.

2.1.8. Perform euthanasia of mice by cervical dislocation and record as dead for statistical analysis.

2.2. Evaluation of colony forming units (CFU) counts in peripheral organs

2.2.1. Use a sub-lethal bacterial dose (5 x 10^5CFU/mice) on the basis of animal survival results to inoculate animals by the i.cist. route (see subsection 1.2).

2.2.2. Perform anesthesia of animals at 48 h post infection as mentioned in step 2.

2.2.3. Proceed with the 70% ethanol disinfection of the chest and withdraw 600-700 µL of blood by cardiac puncture of the chest cavity using a 25 G needle 0.5 x 16 mm.

2.2.4. Collect the blood in a tube containing 3.8% sodium citrate and store at -80 °C for the later viable bacterial cell counts.

2.2.5. Perform cervical dislocation to sacrifice the animals. Confirm the death recording the absence of heartbeat, after sacrificing the mouse according to all relevant institutional and ethical guidelines.

2.2.7. Lay the mouse in the supine position and use scissors and forceps to proceed with the cut of the fur along the sagittal plane of body. Fix the skin with the fur on the sides of the body with pins.

2.2.9. Cut off the peritoneal membrane using sharp scissors. Use scissors and disposable forceps to excise the organs (e.g., spleen and liver) and put each one in sterile Petri dish with 1 mL of GC broth supplemented with 10% (vol/vol) glycerol.

2.2.10. Safely dispose the mouse body as per IACUC guidelines.

2.2.11. Homogenize the organs mechanically at room temperature with the plunger of a 5 mL syringe for about 2-3 min until a single-cell suspension is formed and transfer it in a tube.

2.2.12 Put the tube with the homogenized tissue sample immediately on the dry ice.

NOTE: Samples can be stored at -80 °C in a 2 mL sterile tube for performing the viable bacterial cell counts evaluation at later points.

2.2.13. Make GC agar plates with antibiotics when required. Dry the plates prior to the use pre-incubating at 37 °C for 2-3 h.

2.2.14. Prepare 10-fold serial dilutions of the samples in GC broth from each homogenized tissue and plate onto GC agar plates. Incubate overnight at 37 °C with 5% CO_2.

3. Preparation of brain tissues for CFU count

3.1. Use a sub-lethal bacterial dose (5 x 10^5CFU/mice) on the basis of animal survival results to inoculate animals by the i.cist. route (see subsection 1.2).

3.2. Perform anesthesia of animals at established infection time as mentioned in step 2.

3.3. Perform euthanasia of animals by cervical dislocation.

3.4. Proceed with the 70% ethanol disinfection of the mouse head.

3.5. Cut off the head of mouse using a large scissors.

3.6. Resect the fur and the skin with the help of small surgical scissors and a fine tipped steel forceps, proceeding towards the top of the skull to be able to clearly see the sutures and to guide the opening of the cranium.

3.7. Insert the tip of a tiny scissors through the *foramen magnum* to open the skull.

3.8. Cut towards the center of the cranium and across the midline of parietal bone to the opposite side of the skull. Gently cut along the lateral rim of the lambdoid suture.

3.8. Lift the cranium starting from the posterior parietal corner and pull it diagonally upward to discover the brain, by using fine tipped forceps.

3.9. Be sure that the brain tissue is not attached to any bone of the head, when the skull is lifted.

3.10. Use disposable forceps to remove any connective tissue between the skull and the brain, to ensure that brain tissue being removed along with the skull.

3.11. Do not allow the brain tissue dry out too much. Place the brain, using disposable forceps, in a Petri dish with 1 mL of GC broth supplemented with 10% (vol/vol) glycerol.

3.12. Homogenize the brain mechanically with the plunger of a 5 mL syringe (see step 2.2.11). Transfer the samples into a 2 mL sterile tube and store -80 °C for the later viable bacterial cell counts evaluation as discussed in step 2.2.

REPRESENTATIVE RESULTS:

Survival of mice infected with *N. meningitidis* wild type and isogenic mutant strains.

The *Neisseria meningitidis* strains used in these representative results are the serogroup C reference strain 93/4286 (ET-37) and its isogenic mutant 93/4286Ω*cssA* obtained by insertional inactivation of the *cssA* gene, coding for the UDP-N-acetylglucosamine 2-epimerase, that maps in capsule synthesis locus[25]. To assess the virulence degree of the *cssA*-defective strain in the present murine model, the lethal dose able to determine the death of 50% of infected animals (LD_{50}) was evaluated. To this purpose three groups of animals were infected intracisternally with doses ranging from 10^4 to 10^6 CFU of the wild type strain 93/4286 and with doses of mutant strain 93/4286Ω*cssA* between 10^7 to 10^9 CFU. Generally, the reduction of clinical parameters (e.g., body weight and temperature) and the increasing of mortality rate happened within the first 72 h after the infection. The LD_{50} for the wild type strain corresponded to the meningococcal challenge of 10^4 CFU, whereas the mortality rate with the dose of 10^5 CFU was equal to 83.4% and with 10^6 CFU of 100% (**Figure 1A**). Conversely, to obtain the LD_{50} for the mutant strain 93/4286Ω*cssA*, it has been necessary a dose of 10^8 CFU (**Figure 1B**), an amount of 10,000 folds higher compared to wild type strain.

Evaluation of *N. meningitidis* viable CFU in the mouse brain tissues.

To follow the kinetics of infection in the brain tissue of infected animals, a time course assay was performed with wild type or *cssA* mutant strain[25]. After the i.cist. injection with $5x10^5$ CFU of 93/4286 or 93/4286Ω*cssA* strains, there was a rapid increase of wild type bacteria in the brain tissue reaching the highest numbers at around 24 h post infection (**Figure 2A**); conversely in the brain of mice challenged with the isogenic *cssA*-defective mutant, the viable counts dropped progressively over time until to 2.026 log CFU ± 1.774 72 h post infection (**Figure 2A**). The experiment has shown that 33.3% of the mutant challenged mice showed bacterial clearance

from the infection site, whereas infection with wild type strain was never eradicated from the brain of animals.

Evaluation of meningococcal load in spleen and liver 48h post-challenge.

This experiment was performed to evaluate the clearance of bacteria from infected mice 48 h post-challenge in peripheral organs. To this aim, two groups of mice were infected with 5 x 10^5 CFU of either 93/4286 or 93/4286Ω*cssA* strains, and bacterial viable counts were evaluated in the spleen and liver of infected mice[25] (**Figure 2B**). Following 48 h from the meningococcal injection, the *cssA*-defective mutant was completely cleared in spleen and liver, while the animals infected with wild type strain exhibited a persistent systemic infection framework. The mean values of CFU after 48 h were indeed still 3.212 log CFU ± 3.354 and 6.949 log CFU ± 1.37 in the spleen and liver, respectively. The difference in bacterial loads in the liver tissue of two animal groups was statistically significant (with a P<0.001).

FIGURE AND TABLE LEGENDS:

Figure 1: Survival of mice infected with 93/4286 wild type or *cssA*-defective *N. meningitidis* strains. (**A**) Three groups of Balb/c mice (n= 6/dose) were infected i.cist. with 10^4, 10^5, and 10^6 CFU/mouse of the wild type strain 93/4286 and (**B**) with 10^7, 10^8, and 10^9 CFU/mouse of the isogenic *cssA*-defective mutant. Mice were monitored for a week, and survival was recorded. Results are expressed as percent survival at different doses over time, the log rank p value was < 0.05 for mice infected with the wild type strain. This figure has been modified from Colicchio et al[25].

Figure 2: Evaluation of bacterial loads over time in mice inoculated with the 93/4286 or 93/4286Ω*cssA* strains. (**A**) Time course of bacterial loads in the brain tissues following i.cist. infection. Two groups of Balb/c mice (n=20/group) were infected by the i.cist. route with 5 x 10^5 CFU of either the wild type strain 93/4286 or the *cssA*-defective mutant. Animals were sacrificed 4, 24, 48, and 72 h after infection. Brains were harvested, mechanically homogenized in GC medium, and viable counts were determined. Results are expressed as mean ±SD log of CFU numbers per organ at different time points after inoculation. Asterisks indicate statistical significance (**, P<0.01). (**B**) Bacterial loads over time in spleen and liver. Two groups of Balb/c mice (n=5/group) were infected i.cist. with 5x10^5 CFU of either the wild type strain 93/4286 or the *cssA*-defective mutant. Animals were euthanized 48 h after infection. Spleens and livers were harvested, mechanically homogenized, and viable counts were determined. Results are expressed as log CFU numbers per organ. Horizontal bars indicate mean logs of bacterial titers. Asterisks indicate statistical significance (***, P<0.001). This figure has been modified from Colicchio et al[25].

DISCUSSION:

In this study, we describe an experimental protocol to induce meningococcal meningitis in adult mice by i.cist. inoculation of meningococcal bacteria. To our knowledge, no other model of meningococcal meningitis has been developed in laboratory mice infected by i.cist. route; in the past, this way has been explored to provide models of meningococcal meningitis in both rat[31] and rabbit[32]. It is well-known that the highest rate of meningococcal disease is found between

young children, adolescents, and young adults[33-35]; for this reason, in our meningitis mouse model, instead of focusing on neonatal or infant animals, 8-week-old immunocompetent animals were employed.

In our experimental model, we decided to use i.cist. *inoculum* as it ensures the releasing of the meningococci directly into *cisterna magna* so facilitating bacterial replication in the CSF. This route of inoculation is physiologically more accessible[28] and less traumatic than the intracranial subarachnoidal route, already used for the development of meningitis due to *Streptococcus spp.*[36,37]. Although it does not represent the natural way of infection of meningococcus, the injection of bacteria in this area was instrumental for the induction of meningococcal meningitis, as shown by mouse survival, bacteria loads, clinical parameters, and also by histological analysis[24-26]. Interestingly, reference strain 93/4286 induced meningitis with histopathologic features mimicking those observed in human disease[24-26].

To establish a standardized murine infection and to guarantee the safety of researchers, it was preferred to start from a titrated frozen stock of bacteria rather than a fresh bacterial growth[24,25,30,38], moreover, we decided to use a sub-lethal dose of live meningococci with the aim to limit a rapid fatal outcome and permit the development of brain damage[2,24-26]. Nevertheless, to determine an appropriate infectious dose for different strains, preliminary experiments must be performed. In the present study, we tested a serogroup C reference strain 93/4286 and an isogenic mutant strain 93/4286Ω*cssA* with a dose of 5 x 10^5 CFU/ mice.

Although this model does not mimic the initial phases of colonization and invasion of meningococcus, the bacterial isolate grows well not only in the CSF, but it is also able to remain in the spleen and liver compartments. During the hypoferremic phase of neisserial infection, most of heme-derived iron remains combined with liver ferritin. As ferritin can be used meningococci to obtain iron[39], the liver constitutes a target organ for bacterial replication.

In this experimental procedure, the inbred Balb/c mouse strain was used in replacement of the outbred CD-1 strain that was originally employed to develop the meningococcal meningitis model[24]. Outbred mice were characterized by a wide genetic variability that may be more appropriate to reveal several effects in a variable cohort such as human population[40,41]. However, this variability needs a higher sample size to obtain sufficient statistical significance and may interfere with the standardization of procedures and targeted studies.

Despite the narrow host range of meningococcus, to ensure the replication of bacteria in murine host and enhance the virulence of meningococci, iron dextran was administered to animals before the infection[6,14]. Finally, compared to other experimental model of meningitis, animals were not treated with any antibiotics, to not affect the course of disease and/or profile of the inflammatory response[26].

To date, our studies have highlighted that the i.cist. model is functional to induce both meningitis and invasive meningococcal disease compared to i.p. or i.n. models of infection, which are characterized by the occurrence of sepsis and bacteremia before meningitis is established[10-17].

Therefore, this infection model based on the induction of meningitis in murine host, could be useful not only to evaluate innovative therapeutic strategy to prevent bacterial replication directly into CSF but also to analyze the efficacy of possible passive immune therapy against human pathogens.

However meningococcal infection is a multistep process, that includes nasopharynx colonization, access to bloodstream, crossing of the blood brain barrier and finally uncontrolled proliferation in the CSF, our model only reproduces some aspects of the meningococcal infection, in order to subvert in part these limitations transgenic animal model may be useful to mimic the human pathogenesis of meningococcal disease.

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DISCLOSURES:

The authors have nothing to disclose.

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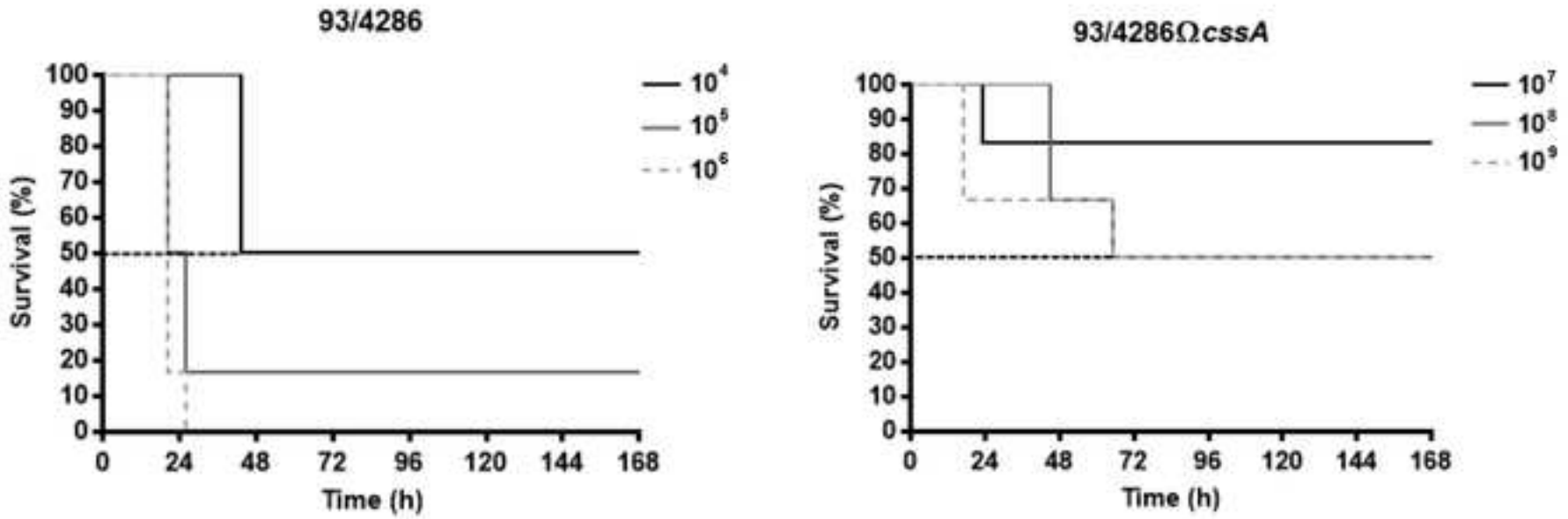
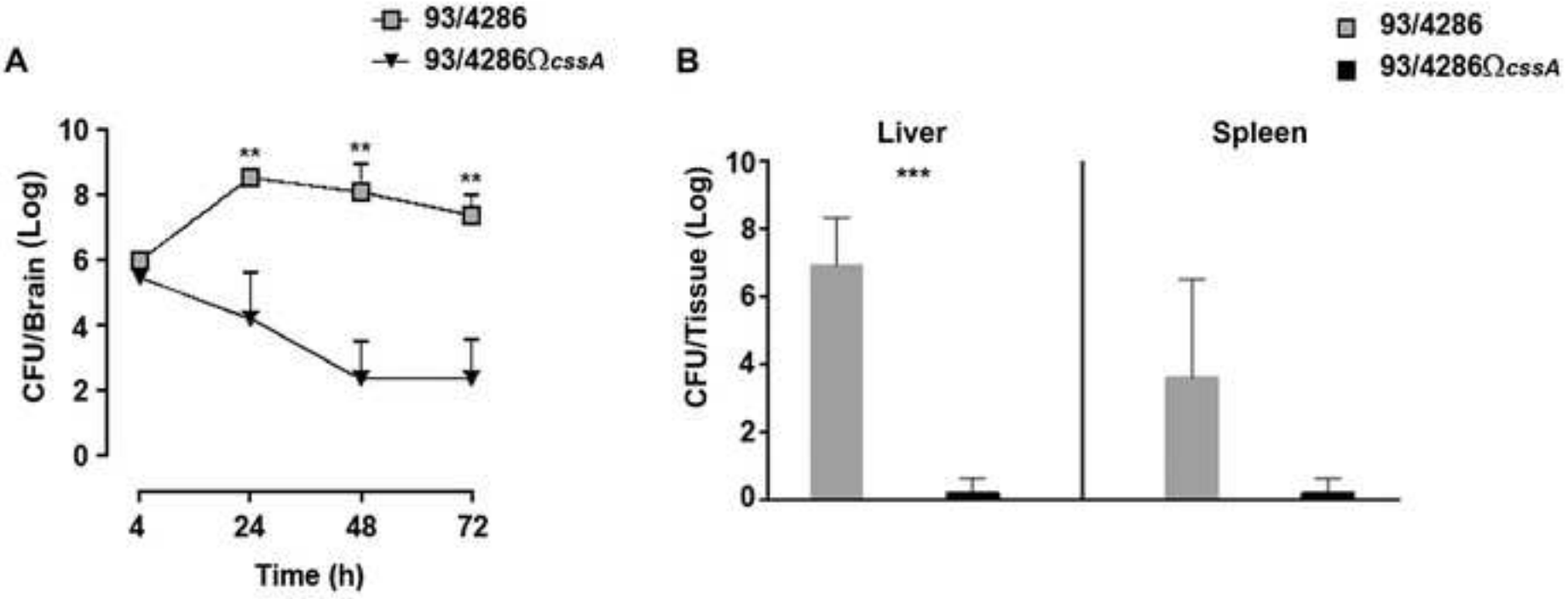
Figure



Figure



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1,8 Skirted Cryovial With external thread	Starlab	E3090-6222	
50ml Polypropylene Conical Tube	Falcon	352070	30 x 115mm
Adson Forceps	F.S.T.	11006-12	Stainless Steel
Alarm-Thermometer	TESTO	9000530	
BactoTM Proteose Peptone	BD	211693	
BD Micro Fine syringe	BD	320837	U-100 Insulin
BD Plastipak syringe 1ml 25GA 5/8in	BD	300014	05x16mm
BD Plastipak syringe 5ml	BD	308062	07 x 30mm
BIOHAZARD AURA B VERTICAL LAMINAR FLOW CABINET	Bio Air s.c.r.l.	Aura B3	
BioPhotometer	Eppendorf	Model #6131	
Bottle D	Tecniplast	D	Graduated up to:400ml, Total Volume 4
C150 CO2 Incubator	Binder	9040-0078	
Cage Body Eurostandard Type II	Tecniplast	1264C	267x207x140mm, Floor area 370cm2
Cell Culture Petri Dish With Lid	Thermo Scientific	150288	Working Volume: 5mL
Centrifuge	Eppendorf	Microcentrifuge 5415R	
Cuvetta semi-micro L. Form	Kartell S.p.A.	01938-00	
di-Potassium hydrogen phosphate trihydrate	Carlo erba	471767	
di-Sodium hydrogen phosphate anhydrous ACS-for analysis	Carlo Erba	480141	g1000
Diete Standard Certificate	Mucedola s.r.l.	4RF21	Food pellet for animal
Dumont Hp Tweezers 5 Stainless Steel	F.S.T. by DUMONT	AGT5034	0,10 x 0,06 mm tip
Electronic Balance	Gibertini	EU-C1200	Max 1200g, d=0,01g, T=-1200g
Eppendorf Microcentrifuge tube safe-lock	Eppendorf	T3545-1000EA	

Erythromycin	Sigma-Aldrich	E-6376	25g
Extra Fine Bonn Scissors	F.S.T.	14084-08	Stainless Steel
Filter Top (mini- Isolator), H-Temp with lock clamps	Tecniplast	1264C400SUC	
GC agar base	OXOID	CM0367	
Gillies Forceps 1 x2 teeth	F.S.T.	11028-15	Stainless Steel
Glicerin RPE	Carlo Erba	453752	1L
Graefe Forceps	F.S.T.	11052-10	Serrated Tip Width: 0.8mm
Inner lid	Tecniplast	1264C116	
Iron dextran solution	Sigma-Aldrich	D8517-25ML	
Ketamine	Intervet		
Microbiological Safety Cabinet BH-EN and BHG Class II	Faster	BH-EN 2004	
Microcentrifuge tubes 1.5ml	BRAND	PP780751	screw cap PP, grad
Mouse Handling Forceps	F.S.T.	11035-20	Serrated rubber; Gripping surface:15 x
Mucotit-F2000	MERZ	61846	2000ml
Natural Latex Gloves	Medica	M101	
New Brunswick Classic C24 Incubator Shaker	PBI international	C-24 Classic Benchtop Incubator Shaker	
Petri PS Dishes	VWR	391-0453	90X14.2MM
Pipetman Classic P20	Gilson	F123600	2-20microL
Pipetman Classic P200	Gilson	F123601	20-200microL
Pipetman Classim P1000	Gilson	F123602	200-1000microL
Polyvitox	OXOID	SR0090A	
Potassium Chloride	J.T. Baker Chemicals B.V.	0208	250g
Potassium Dihydrogen Phosphate	J.T. Baker Chemicals B.V.	0240	1Kg
PS Disposible forceps	VWR	232-0191	
Removable Divider	Tecniplast	1264C812	
Round-Bottom Polypropylene Tubes	Falcon	352063	5ml

Sodium Chloride	MOLEKULA	41272436	
SS retainer and Polyester FilterSheet	Tecniplast	1264C	
Standard Pattern Forceps	F.S.T.	11000-12	Stainless
Stevens Tenotomy Scissors	F.S.T.	14066-11	Stainless Steel
Surgical Scissor - ToughCut	F.S.T.	14130-17	Stainless
Touch N Tuff disposible nitrile gloves	Ansell	92-500	
Ultra Low Temperature (ULT) Freezer	Haier	DW-86L288	Volume= 288L
Wagner Scissors	F.S.T.	14070-12	Stainless Steel
Xylazine	Intervet		

150ml, 72x72x122mm

20 mm

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Response to Editor Comment

Comment [A1]: Citation?
R. Now the citations were added in the text (Line 94).

Comment [A2]: Citation?
R. Now the citations were added in the text (Line 99).

Comment [A3]: Please ensure that the protocol describes all the steps needed to perform your experiment and all specific details are included.
Also, please ensure that the protocol makes a cohesive story from one step to the next. Presently there is a missing link from one subheading to the next.
R. We thank the Editor for the suggestion. The Protocol is now described in detail step by step and all specific details are included (Line 135).

Comment [A4]: Reworded for clarity please check. Please use 1.1. as example and bring out clarity throughout the protocol section.
R. The Title of section 1.1 has been modified for clarity: "Infection of mice with N. meningitidis Serogroup C strain" (Line 150).

Comment [A5]: Do you snap freeze after this step? How long can you store the bacteria for good results?
Comment [A6]: Please include a note stating that it is better to use fresh bacterial culture, but frozen stocks were made because of to ease the experiment. Then please detail the reason in the discussion section.
R. As indicated by the Editor, a note was added to indicate the use of frozen cultures, the storage time and finally that it is better to use fresh bacterial culture, but frozen stocks were made because of to ease the experiment (Lines 164-166).

Comment [A7]: Please include the reason of growing bacteria in iron dextran substrate.
R. A note was added to indicate the use of iron dextran in the preparation of the bacterial suspension for the i.cist. inoculum (Lines 173-174).

Comment [A8]: Please include the volume.
R. Now the volume used for the preparation of the bacterial suspension has been reported (Line 170).

Comment [A9]: For how long? Also, please include how do you determine the CFU.
R. Now in the text the incubation time and the procedure for the assessment of viable CFU are specified (Lines 177-179).

Comment [A10]: What is the CFU in this case?
R. The note has the aim to better explain the preparation of bacterial inoculum to test in the mice. It is only an example. Furthermore, in our opinion this note is not appropriate in this protocol's step. The note refers to the preparation of bacterial dose for the bacterial suspension to inoculate by i. cist. route. So, the note has been moved after the step 1.2.8 (Lines 227-234).

Comment [A11]: How much bacteria are injected per animal?
R. The number of bacteria injected in each murine host is established by the researcher on the basis

of bacterial dose to be tested.
Is it based on the body weight?
R. No it is independent. Also, as reported above, this Note is not appropriate in this protocol's step. The note refers to the preparation of bacterial dose for the bacterial suspension to inoculate by i. cist. route. So, the Note has been moved after the step 1.2.8.

Comment [A12]: Some of the steps have been moved around to bring out better clarity.
R. We thank the editor for the changes, to make the protocol clearer.

Comment [A13]: Please mention what should be the weight and temperature before the start of the experiment. (Around 20 g and 37 degrees?).
R. As indicated by the Editor, a Note was added to indicate the weight and average body temperature of mice in physiological conditions (Lines 202-203).

Comment [A14]: What is the solvent in this case?
R. Iron dextran is dissolved in Phosphate saline buffer (Lines 206-207).
What is the size of the needle and syringe used?
R. The size of the needle used has now been specified (25-gauge needle 0.5 x 16 mm) (Line 207).
Do you disinfect prior to injection?
R. For disinfection 70% Ethanol was used as reported (Line 205).
Any specific place of injection?
R. The intraperitoneal inoculum site has now been specified in the Note (Lines 210-211).

Comment [A15]: This is moved here to bring out clarity.
R. We agree with the Editor for this change (Lines 212-213).

Comment [A16]: This sentence is moved here to bring out clarity. Also, before this please include a sentence stating that the syringe was loaded with the bacterial suspension first. What is the syringe size? what is the bacterial count used in what volume?
R. The sentence was now corrected. The number of bacteria injected in each murine host is established by the researcher on the basis of bacterial dose to be tested.

Comment [A17]: What is the CFU in this case?
R. The note has the aim to better explain the preparation of bacterial inoculum to test in the mice. It is only an example.

Comment [A18]: How much bacteria are injected per animal?
R. The number of bacteria injected in each murine host is established by the researcher on the basis of bacterial dose to be tested.
Is it based on the body weight?
R. No it's independent.

Comment [A19]: Do you use iodine based scrub as well?
R. No, we use 70% Ethanol as reported (Line 236).

Comment [A20]: Injection area? Do you perform any surgery in this case?
R. We don't perform surgery, we perform the intracisternal injection with the help of the needle, as described in detail in the note of 1.2.10 section (Lines 243-250).

Comment [A21]: What is the established CFU (Please provide a number) and how was this established? Please list the steps for the same.
R. In the previous Note (lines 227-234) we describe the procedure to establish the CFU starting from post thawing bacterial titer. The established CFU for the i.cist. inoculum depends to the bacterial dose to be tested.

Comment [A22]: How is this identified/made?
R. The identification of the inoculation site was made by palpation and it was now described in detail in the Note of 1.2.10 section (Lines 243-250).

Comment [A23]: We cannot have two notes following each other, the above note moved to the section above.
R. As indicated now in the Manuscript there are not two consecutive Notes.

Comment [A24]: What is injected in case of control animals?
R. In the development of the infection model the control mice were inoculated only with GC broth supplemented with iron dextran. See step 1.2.10 (Line 239).

Comment [A25]: Do you perform the animal survival and CFU count at this stage of the experiment on different animals? If yes, then please approve the sentence added here. Else please include a note to clarify step 1 and step 2 are different experiments.
R. As stated in the steps 2.1.1 and 2.2.1 the infection of mice by i.cist. route is essential for both animal survival and evaluation of CFU counts in peripheral organs (that are two independent experiments). Therefore, the step 1 represents the initial step for both experiments. So, the step 1 and step 2 cannot be considered as different experiments.

Comment [A26]: Only cervical dislocation cannot be used for euthanasia. Please include CO2 overdose as well.
R. Because our in vivo experimentation was entirely performed in laminar flow cabinet it is difficult use CO2 overdose for the euthanasia. In addition, the euthanasia of mice was always humanly performed after anesthetization followed by cervical dislocation.

Comment [A27]: Please include the number of days you perform your experiment for.
R. As suggested now the number of days was specified (Lines 279-280).

Comment [A28]: Do the mice die on its own or do you perform euthanasia on selected group?
Comment [A29]: Please include the chosen time point in your case and why this was chosen. E.g. The chosen time point in our case was……
R. The survival assay is based on recording the survival of animals infected with different doses in order to identify the lethal dose for 50% of animals (LD50) for a selected strain. However, for clarity the sentence was rewritten (Lines 291-292).

Comment [A30]: Also, not clear why is anesthetization performed followed by the euthanasia? If this just describes the end point, please reword as: After one week, euthanized all the mice ……
R. For clarity, the sentence was rewritten and a note was added to section 2.1.5 (Lines 287-288).

Comment [A31]: Please include the dose used in your experiment.

R. Now the dose used was added, 2.2.1 section (Line 302).

Comment [A32]: Again, please provide the time point established in your case.
R. Now, the time point was added in the sentence (Line 305).

Comment [A33]: Do you place this on ice?
R. It is not necessary, after the homogenization of the sample, the tube with the homogenized biological sample was immediately placed on dry ice. Now the procedure has been inserted in the text (Line 326).

Comment [A34]: Brian dissection can be included at this stage itself, since you are only dissecting spleen and liver here. If using different mouse for both dissections, please include a note stating why the same mouse cannot be used.
R. The evaluation of CFU counts in peripheral organ and the evaluation of CFU counts in the brain tissue are two independent experiments, where the counts in peripheral organs were evaluated in a single time: 48 hours post infection; whereas the evaluation of CFU counts in the brain tissue has been performed in a time course (4, 24, 48, 72 hours post-infection), as reported in Figure 2A. So, the brain dissection cannot be mentioned at this point of the protocol.

Comment [A35]: Do you wash before homogenization?
R. No we don't wash.
Do you perform this step at room temperature or on ice?
R. We perform this step at room temperature and now it is indicated in the text (Line 330).

Comment [A36]: Don't you perform a serial dilution in this case?
R. Yes, we perform a serial dilution, now it is specified in the text (Line 341).

Comment [A37]: Please include the calculation of how to perform the CFU count.
R. After an over night incubation of GC agar plated, it is possible to observe the growth of colonies that is possible to count with naked eyes. This is a well standardized method.

Comment [A38]: This section can be merged with the section above from step 7-14.
R. In our opinion, the evaluation of CFU counts in the brain tissue is an independent experiment from the evaluation of CFU counts in the peripheral organs, so it is reasonable reports this step in a separate section.

Comment [A39]: The control mentioned in the protocol is GC broth, however in the result data for isogenic mutant is presented. Please include a note stating that the reference strain and its isogenic mutant is used for the experiment.
R. In the step 1.2.10 now was reported the use of wild type and isogenic mutant strain for the in vivo infection.

Comment [A40]: Presently the discussion is majorly the repetition of the results. Please revise the discussion to focus on the protocol explicitly covering the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique

d) The significance with respect to existing methods
e) Any future applications of the technique

R. All the indications provided by the Editor was now added.

Comment [A41]: No data for this is shown in the manuscript. Please include a citation or tone down the claims.

R. The citations were now included in the text (Line 470).

Comment [A42]: Not shown in the manuscript.

R. Citations related to these experiments have now been included in the text (Line 472).

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Title: Virulence Traits of a Serogroup C Meningococcus and Isogenic *cssA* Mutant, Defective in Surface-Exposed Sialic Acid, in a Murine Model of Meningitis

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