The Effects of Sodium Chloride on *Haemophilus influenzae* Growth

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Abstract ***Haemophilus influenzae,*** **the gram-negative facultative bacterium, infects humans and causes a wide variety of respiratory tract diseases. In this study the growth characteristics of *H. influenzae* were examined using different growth media including Brain Heart Infusion, Luria Broth, and Chemical Defined Medium. In addition a series of sodium chloride concentrations were examined in each medium to investigate ideal growth conditions for *Haemophilus* cultivation. Our data suggested that *H. influenzae* growth is affected by the presence and concentration of sodium chloride (NaCl) in the complex-nutrient medium Luria Broth. The growth of *H. influenzae* in Chemical Defined Medium and Brain Heart Infusion was also monitored. Chemical Defined Medium represents a minimum-nutrient condition and Brain Heart Infusion represents a rich-nutrient condition. Our results demonstrated that when grown in LB *H. influenzae* grows the best with a 0.5% NaCl concentration; there is no significant difference in the growth rate of *H. influenzae* in the presence or absence of additional sodium chloride in both Brain Heart Infusion and Chemical Defined Medium.**

Introduction

Haemophilus influenzae is a fastidious gram-negative bacterium that usually colonizes the human nasopharynx asymptomatically. Approximately 20-40% of adults carry the opportunistic pathogen *H. influenzae*, with young children being colonized at a much higher rate, of up to 85% (Kilian et al. 1972; Kuklinska and Kilian 1984). *H. influenzae* manifests itself in a wide range of clinical diseases including, but not limited to, otitis media, sinusitis, bacterial meningitis, pneumonia, bacteremia, epiglottitis, osteomyelitis, cellulitis, and conjunctivitis (Farley et al. 1992). Typically *H. influenzae* strains are classified according to the presence or absence of a capsule structure. Encapsulated *H. influenzae* strains (type b) usually cause more invasive diseases, such as meningitis, pneumonia, and bacteremia. Strains lacking a polysaccharide capsule are classified as Nontypable (NTHi). Nontypeable *H. influenzae* is responsible for less invasive infections like otitis media, sinusitis, and bronchitis (St. Geme 1993). The growth of *H. infleunzae* requires X and V factor supplements (Evans et al. 1974). Nicotinamide Adenine Dinucleotide (NAD) has always served as an excellent V factor to support *H. influenzae* growth (Evans and Smith. 1972). Hemin has consistently been used as an X factor supplement, as it is an essential source of iron and porphyrin for several pathogenic bacterial species *in vitro*.

This study examines whether additional NaCl will have a positive or negative effect on the growth of *H. influenzae* in three growth media, Luria Broth (LB), Chemical Defined Medium (CDM), and Brain Heart Infusion (BHI). Supplemental NaCl concentrations of 1% and 0% were examined in LB, CDM, and BHI, the typical laboratory growth media for *H. influenzae*. In order to further evaluate the effect of NaCl on *Haemophilus* growth in LB, the broth was supplemented with additional amounts of NaCl (0%, NaCl, 0.5% NaCl, 1% NaCl, 5% NaCl, 10% NaCl) and the growth of *H. influenzae* was measured. LB is composed of tryptone, an enzymatic digest of protein that provides amino acids for the bacteria, yeast extract and sodium chloride (MacWilliams and Liao 2006). In this study, we addressed the question whether the additional NaCl with have a positive or negative effect on the growth of *H. influenzae*.

Materials & Methods

Bacterial stains and culture media

The bacterial strains used in this study were *H. influenzae* Rd (ATCC51907) and encapsulated Egan (ATCC9795), which represent both laboratory and clinical isolates. The growth of *H. influenzae* was carried out in three different media: Brain Heart Infusion (BHI), Luria Broth (LB), and Chemical Defined Medium (CDM).

CDM was prepared as ollowing: 191 mL of RPMI 1640 with L-glutamine and 25 mM HEPES, 10 mL of a 2-mg/mL uracil solution dissolved in 0.1 N NaOH and 20 mL of a 20 mg/ml inosine solution dissolved in deionized water and filter sterilized (Colemen et al. 2003). LB and BHI broth were prepared according to manufacture's instruction.

Preparation of X and V Factors

The Nicotinamide Adenine Dinucleotide used in this experiment was 2μg ml^{-1}. Hemin stock was prepared by dissolving 0.2 g of L-Histidine in 200 mL of deionized water and then adding 0.2 g of Hemin HCL and 4 mL of 1

N NaOH and steaming over a boiling water bath for 5-10 minutes to create a soluble mixture (Coleman et al. 2003). The solution was then cooled to room temperature, filter sterilized, and placed in a foil-covered bottle. All the growth media were freshly prepared and supplemented with X and V factor before bacterial inoculation.

Bacterial Growth Curves

The bacterial growth curves were performed in the following manner: overnight cultures of *H. influenzae* in the 37°C shaking at 210 rpm were inoculated 1/50 into a spec tube with freshly prepared growth medium, and the bacterial density were measured every 30 minutes by using the spectrophotometer at Optical Density of 600 nm (Thermospectronic: Genesys 20). The growth curve data were all based on three independent experiments unless otherwise indicated.

Statistical Analysis

All of the growth curves were graphed with standard deviation. The growth rate was statistically analyzed using a one-way ANOVA test, and the p values were determined to evaluate the significant difference in *H. influenzae* multiplication under three different growth media (BHI, LB and CDM) in the presence or absence of sodium chloride.

Results

H. influenzae strain Rd was grown in CDM along with either a 1% NaCl concentration or no addition of NaCl. There was no significant difference ($p>0.01$) in their growth curves (Figure 1, Part A), whether it was in the log phase or stationary phase of the growth curve. The same was also true when *Haemophilus* was tested in BHI under the same NaCl conditions (Figure 1, Part B). However, when LB was used as a nutrient-medium a significant difference in the growth rate was observed in the presence of 1.0% NaCl (Figure 1, Part C). The difference was noticeable even during the early log phase of the growth.

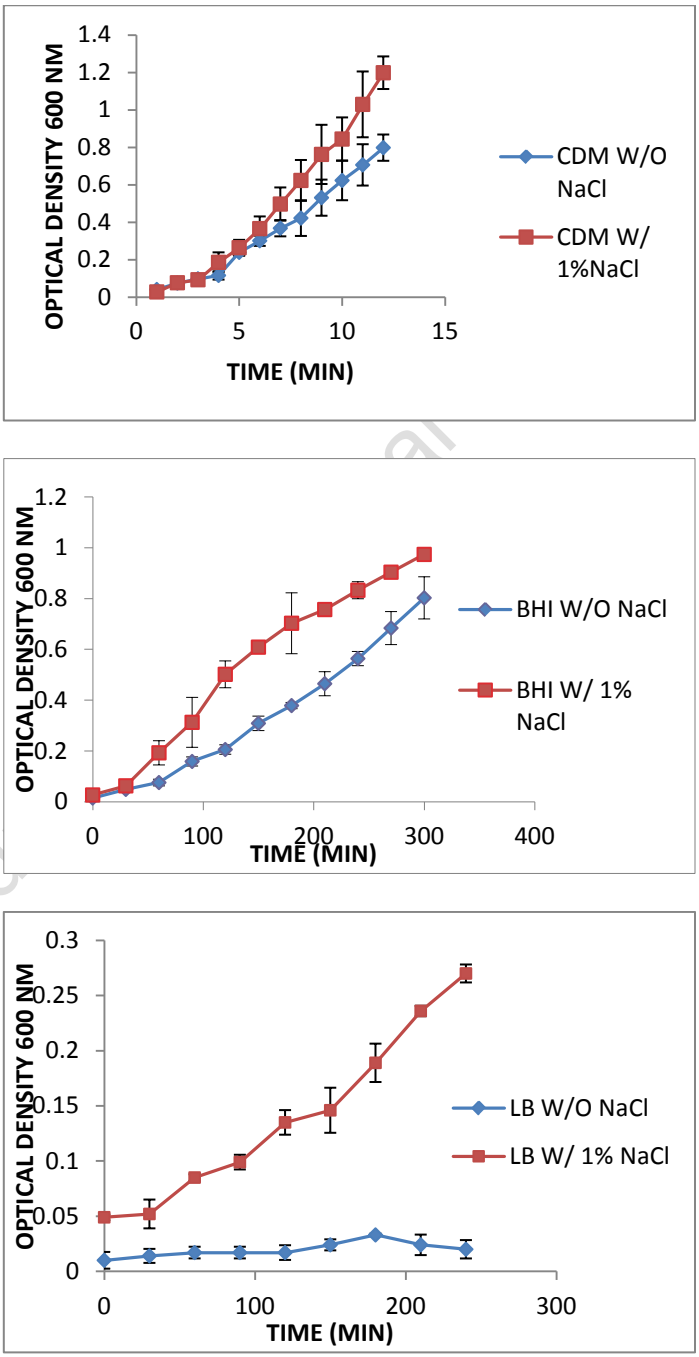

Figure 1 (A - *top*) Growth characteristics of *Haemophilus influenzae* in Chemical Defined Medium in the absence of salt and with the presence of a 1% NaCl concentration were compared. Each data set represents three independent experiments in triplicate. (B - *center*) Growth characteristics of *Haemophilus influenzae* in Brain Heart Infusion under 1% NaCl concentration and with no salt. (C - *bottom*) Growth characteristics of *Haemophilus influenzae* in Luria Broth under 1% NaCl concentration and with no salt.

To further evaluate *H. influenzae* growth in LB an overnight culture of *H. influenzae* strain Rd was inoculated into LB along with one of the NaCl concentrations test-

ed in this study (10% NaCl, 5% NaCl, 1.0% NaCl, 0.5% NaCl, 0.0% NaCl). *Haemophilus* growth was measured using the spectrophotometer. Growth curves in the presence of varying sodium chloride concentrations were compared (Figure 2, part A). In the mid-log phase of the growth curve, *Haemophilus* density doubled at a 0.5% NaCl concentration. The 1.0% NaCl concentration did not support the growth of *H. influenzae* as efficient as the 0.5% NaCl concentration; however, it was noticeably more conducive to the growth of *H. influenzae* in comparison to the 10%, 5%, and 0% NaCl concentration. An ANOVA (Analysis of Variance) test was performed and the results of the statistical models clearly demonstrate that *Haemophilus* growth was highest with a 0.5% NaCl supplement ($P<0.0001$). Therefore, *H. influenzae* significantly improved its growth rate with additional 0.5% NaCl in LB medium.

The exceptional growth of *H. influenzae* in LB with a 0.5% NaCl concentration is further evident in Figure 2 (part B) where the mean growth rates of *Haemophilus* in the varying NaCl concentrations is graphed. *Haemophilus* density with a 0.5% NaCl concentration is almost five times more than that at a 0% NaCl concentration and more than twice that at a 1% NaCl concentration.

To further confirm our data encapsulated *Haemophilus* clinical strain Egan was also tested in the above media. Similar results were observed (Data not shown).

Discussion

The ability of *H. influenzae* to grow *in vitro* requires the media to be supplemented with Hemin and NAD (Bergeron et al. 1987), because the bacterium lacks the enzymes for the synthesis of both Hemin and NAD. The purpose of this study was to compare the three growth media and examine sodium chloride as an additional component to support *Haemophilus* growth. Sodium chloride is the most commonly used salt in laboratories to mimic physiological osmotic conditions in environment and inside cells, for example the human body has about a 0.9% NaCl concentration (Dötsch et. al. 2009). To our knowledge the effect of additional varying NaCl concentrations has not been previously examined in *Haemophilus* and any other fastidious pathogenic microorganisms, which use human body as their host for infection.

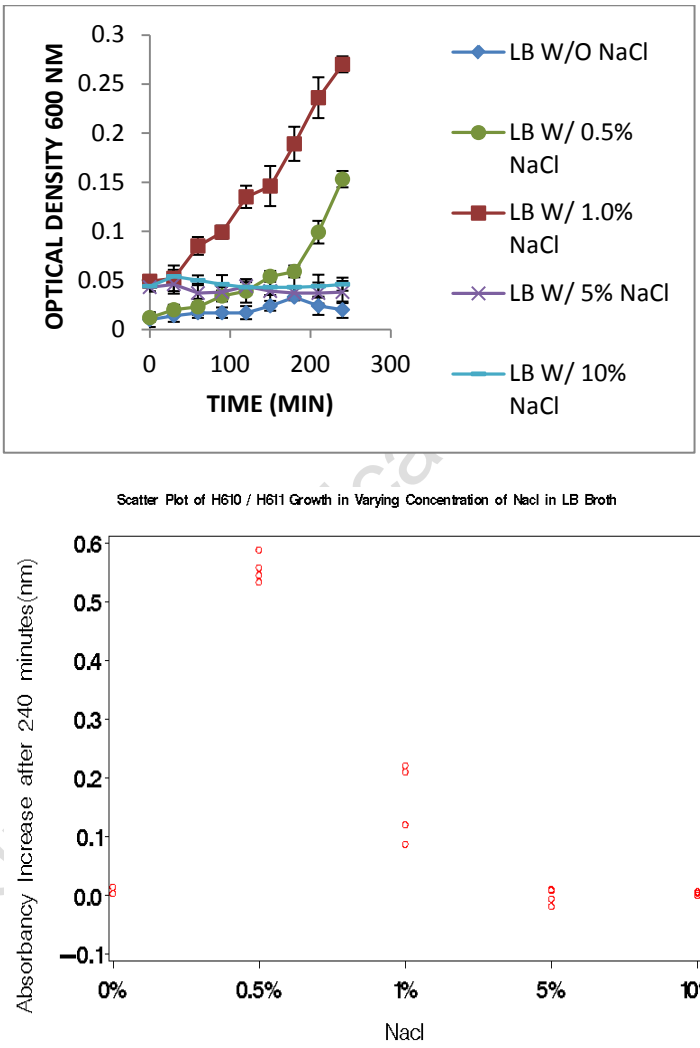

Figure 2 (A - *top*) Growth characteristics of *Haemophilus influenzae* in Luria Broth under 10% NaCl concentration, 5% NaCl concentration, 1% NaCl concentration, 0.5% NaCl concentration, and with no salt. (B - *bottom*) Scatter plot of *Haemophilus influenzae* growth in LB with varying concentrations of NaCl. The 0.5% NaCl concentration shows the most growth.

The nutrient rich medium BHI is expected to contain a high concentration of growth factors that are released by the enzymatic digestion of brain and heart tissues in the production of the medium. In the nutrient rich medium BHI, *Haemophilus* is able to grow at analogous rates with either a 1% NaCl concentration or no additional NaCl. On the contrary, the nutrient–limiting medium CDM contains the minimal nutrients that *Haemophilus* requires for growth, *H. influenzae* demonstrated a lower growth rate in CDM than in BHI, and, the addition of a 1% NaCl concentration was not sufficient in subsidizing the nutrient requirement for the growth of *Haemophilus.* We did not observe any notable difference in the growth of *Haemophilus* grown with a 1% NaCl concentration compared to

no additional NaCl.

When a 1% NaCl concentration and no additional salt were examined in LB there was a substantial difference in the growth of *Haemophilus*. The bacteria multiplied much more rapidly in LB with a 1% NaCl concentration than they did in the absence of NaCl. After comparing *Haemophilus* growth in CDM, BHI, and LB we focused on examining five different sodium chloride concentrations, 10% NaCl, 5% NaCl, 1% NaCl, 0.5% NaCl, and 0% NaCl (possible environmental salt conditions), and their effect on *H. influenzae* multiplication in LB, as LB medium was the more conducive to *H. influenzae* growth with an additional 1% NaCl concentration.

When Luria Broth was used as a complex-nutrient medium under standard laboratory conditions to support the growth of *H. influenzae*, all five sodium chloride concentrations were examined. The growth of *Haemophilus* was measured at a much lower rate when a 10%, 5%, and 0% NaCl concentration were used as an additional supplement. *Haemophilus* grew noticeably better in a 1.0% NaCl concentration as compared to 10%, 5%, and 0%, however, a vastly higher rate of growth was obtained in the presence of 0.5% NaCl as compared to 10% NaCl, 5% NaCl, 1.0% NaCl and 0% NaCl.

H. influenzae is a human pathogen and, as such, has evolved to possess attributes that facilitate its survival and prosperity in the human upper respiratory tract or environments that mimic similar conditions (Hallström and Riesbeck 2010). Due to this fact and the data from our study, we can hypothesize that *H. influenzae* will multiple most efficiently in a 0.5% NaCl concentration because, along with the 0.4% NaCl concentration already in LB, it most closely mirrors the conditions of the human body. When a 0.5% NaCl concentration is added to the 0.4% NaCl concentration already present in LB it creates a 0.9% NaCl concentration that is approximately equivalent to that of the human body. Conversely, the growth of *Haemophilus* was measured at a much lower rate when a 10% NaCl concentration (possible environmental osmotic shock conditions) was used as an additional supplement because it was too inconsistent with the conditions of the human upper respiratory tract under which *H. influenzae* has evolved to survive and cause infection.

In conclusion, the effects of varying NaCl concentrations on different strains of *H. influenzae* growth have been examined. Evidence provided by this study indicated that *Haemophilus* grew most rapidly in BHI, and that *H. influenzae* grows in LB substantially better when there is a 0.5% NaCl concentration. In contrast, under nutrient-limiting conditions and rich-nutrient conditions the addition of 1% NaCl is negligible as there is no significant difference in the growth of *H. influenzae*. Our results also have implications for other fastidious bacterial species that are human-pathogens and have similar growth requirements.

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