Anti-Microbial Susceptibility Patterns of Haemophilus influenzae from Patients in Nakorn Chiang Mai Hospital

Suputra Peerakome, B.Sc. (Med. Tech.)**
Kampol Panas-ampol, M.D.**

Abstract

Nineteen strains of Haemophilus influenzae were isolated from 75 patients at Nakorn Chiang Mai Hospital. The identification was based on morphology, staining property, colonial characteristics, satellite phenomenon and the requirement of both X and V factors. The susceptibility to ampicillin, tetracycline, chloramphenicol, streptomycin and sulfadiazine were determined by using a plate dilution method and the multipoint inculcator. Readings of the minimal inhibitory concentration (M.I.C.) were made after 24 hour incubation at which time there was complete growth of the organisms on the control plate. The results were compared with the known average blood level concentrations in people and classified as sensitive, intermediate or resistant on this basic. We found that all strains had the same susceptibility pattern. They were sensitive to ampicillin, tetracycline and chloramphenicol but resistant to streptomycin and sulfadiazine. The comparison of undiluted inoculum and 0.1 dilution were included in this study. Most of strains showed the same result with a maximum difference of one two-fold dilution.

Introduction

Haemophilus influenzae plays an important etiological role in bacterial meningitis, especially in children under 2 years old. It sometimes produces septicaemia, obstructive epiglottitis, pneumonia(1), severe conjunctivitis, arthritis, laryngitis, endocarditis (2), sinusitis, laryngotracheitis (3), relapsing illness, otitis media, pneumonitis or chronic lung disease (1). It is

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** Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai
often implicated as a "secondary invader" and more than half the meningitis patients have upper respiratory tract infection before contracting meningitis.

**Morphology (3, 4, 5, 6)**

In 1892-1893 (5), Pfeiffer first isolated the organism from the nasopharynx of epidemic influenza patients, mistakenly believing it to be the cause of the influenza. It is a small gram negative aerobic bacillus, 0.5 micron in length and 0.2-0.3 micron in diameter, non-motile. It is pleomorphic, and may appear as coccoid bacilli, short chains, long rods, or large spherical bodies. Morphology depends on age and media. After 6-8 hours, cultures on enriched media appear as coccobacilli, but after longer incubation appear as long pleomorphic rods. In exudates and in 6-18 hours cultures on enriched media, capsules are present. However these autolyse in autolytic enzyme and disappear in older cultures. On Levinthal agar after 24 hours incubation, it appears as a small, round, convex colony with strong iridescence. The iridescence is due to the presence of capsules. On chocolate agar, after 36-48 hours it appears as large colony, 1 m.m. in diameter. On blood agar, it grows poorly or not at all and produces no hemolysis. However it will produce a large colony on blood agar if growing in the vicinity of Staphylococcus, Neisseria, or Pneumococci colonies. These organisms produce a growth factor required by H. influenzae. This phenomenon is called "Satellitism" or "Satellite phenomenon".

**Growth Characteristics (7, 8)**

H. influenzae requires media enriched with X-factor, a heat-stable substance called hemin, extracted from blood, and V-factor, a heat-labile substance DPN or TPN. V factor may be extracted from vegetables, yeasts, whole blood, and is produced by certain bacteria. Identification of the species is generally based on requirement for the growth factors X and V and reaction with specific antisera.

**Transformation (3)**

H. influenzae extracted DNA can transfer type specificity, such as antibiotic resistance to other cells.

**Antigenic Structure**

H. influenzae's capsule, which is important in virulence, possesses polysaccharide antigens. Based on these antigens, 6 serological types, a, b, c, d, e, f are recognized. In capsular swelling (quellung) and carbohydrate precipitin tests, cross-reactions with pneumococci occasionally occur. Most meningitis cases are caused by type b (5).

**Diagnostic Laboratory Tests**

**Specimens**

Specimens may include nasopharyngeal swabs, pus, blood, C.S.F. (3), throat swabs, exudate from eye and ear (1), oropharyngeal exudate (9), tracheal aspirates,
lung aspirates, and exudate from wounds (10). In meningitis cases, organisms can be isolated from C.S.F., throat swabs and in 75% of cases, from the blood.

**Direct Identification**

The specimen is examined by gram stain and cultured on appropriate media. It has been recorded that from C.S.F. from H. influenzae meningitis cases, the organism was found in 88.4% of specimens by gram stain, but only 78.3% of the subsequent cultures were positive (11). If large amounts of organisms are found in the specimen, gram stain, direct typing by capsular swelling on C.S.F. (3) can be done. Precipitin tests may be done for detection of organisms in C.S.F.; a positive result indicates high concentration of specific H. influenzae type b polysaccharides (3).

Selective media for detection of H. influenzae may be prepared by adding antibiotics such as bacitracin or penicillin to culture medium. The media most often used in the laboratory is chocolate agar enriched with hemin and supplement B (yeast extract); but problems arise because gamma streptococci, enterococci, diptherioids, and neisseria may give similar colony patterns, iridescence is hard to see, and supplement B is easily destroyed by heat.

H. influenzae grows excellently on media enriched with red blood cells which have been hemolyzed by heat, such as Levinthal's medium, or hemolyzed by peptic digestion, such as Fildes's medium. Both media are transparent, so iridescence of Haemophilus species can be seen. However these are difficult to prepare.

A new method of isolation is that of placing a saponin-soaked disc on the surface of sheep blood agar. The saponin hemolyzes red blood cells, and splits out di-phosphopyridine nucleotide. This has the same properties as Levinthal's and Fildes's medium, with the advantage of showing hemolysis of the other hemolytic pathogens (12).

**Immunity**

Active immunization can not induce immunity in man. A newborn baby's immunity disappears during the first 6 weeks, after which immunity increases until 3-4 years old when bactericidal activity against virulent strains of H. influenzae occurs. Fothergill and Wright (6) found that animals could be immunized.

**Treatment**

In the past, H. influenzae type b rabbit antiserum had a moderate effect in decreasing the percentage of fatality in patients (to 90% of fatality rate for untreated patients). (3, 5, 6)

Detection of antibody may be done by using H. influenzae type b's capsule as a skin testing agent, with intradermal injection. If the test is positive, after 30 minutes erythema with pseudopodia ce-
cures (6). Now, type-specific rabbit anti-serum is not used in treatment, because it can cause serum sickness. Antibiotics and sulfadiazine are preferred. The present study investigated the current antibiotic susceptibilities of local H. influenzae strains to these antibiotics most frequently used in treatment.

Material and Method

I Bacterial Strains

H. influenzae was isolated from throat swabs of 75 patients at Nakorn Chiang Mai Hospital during the period from November 13, 1969 to January 13, 1970.

Isolation for pure culture

Media

- Chocolate agar

G.C. agar base was used Autoclaved at 15 lbs/in.² for 15 minutes, it was then placed in an 80°C waterbath. By aseptic technique 10% sterile blood was added, and the mixture left at 80°C for 10 minutes. The temperature was then lowered to about 45°C, and 1% supplement B added.

- Blood agar

Blood agar base was autoclaved at 15 lbs/in.² for 15 minutes, cooled it until the temperature was 48-50°C, and 10% sterile blood was added.

Specimens were inoculated on chocolate agar plates. Isolation was based on colonial characteristics. Suspected colonies were picked and inoculated on blood agar plates. A staphylococcus epidermidis, as a source of V factor, was streaked through the inoculum, and after 24 hours incubation, plates were checked for the “Satellite phenomenon”. A gram stain was made to check for the typical gram-negative coccobacilli or gram-negative bacilli.

H. influenzae is different from other species in its requirement of both X and V factor (3,4,5,6). Media used to identify the Haemophilus species were: autoclaved chocolate agar (containing only X factor but no V factor), and trypticase-soy agar (containing neither X nor V factor). If satellite phenomenon occurred around a streak of Staphylococcus epidermidis only on the autoclaved chocolate agar, identification of the species of H. influenzae was confirmed.

II Antibiotics

The antibiotics used in this study were:

- Tetracycline hydrochloride
- Chloramphenicol
- Streptomycin sulfate
- Ampicillin sodium
- Sulfadiazine sodium

Stock Solution of Antibiotics (24)

Antibiotic powder was dissolved in sterile distilled water, and the concentration of tetracycline, chloramphenicol and streptomycin adjusted to equal 1,000 mcg/ml, ampicillin to equal 500 mcg/ml, and sulfadiazine to equal 1,000 mg%.
Stock solutions were stored in 10 ml aliquot portions, frozen at -70°C. Aliquots were discarded after a single use.

**Working Solution of Antibiotics**

Sensitivity testing was done by using a plate dilution method. The stock solutions of antibiotics were used as working solutions.

**Plate Dilution Method**

**Media**

- **Mueller - Hinton chocolate agar**
  
  Mueller-Hinton agar was used as a base. By aseptic technique 10% sterile blood was added at 80°C, and after 10 minutes the mixture was cooled to 45-50°C and the solution of antibiotic or sulfadiazine added. Plates were poured.

- **Levinthal’s broth (1, 22, 23)**

  Mueller-Hinton broth was used as a base. By aseptic technique 10% sterile blood was added at 80°C. After 10 minutes, the mixture was filtered, the supernatant portion collected and finally 1% supplement B added.

**Serial dilution of antibiotics for plate dilution method and preparation of plates.**

Serial dilution of antibiotics for plate dilution method was based on the distribution and concentration of antibiotics in blood and C.S.F., and dose of antibiotic generally administered.

Highest concentrations used of tetracycline, chloramphenicol, streptomycin were 100 mcg/ml; for ampicillin and sulfadiazine they were 25 mcg/ml and 100 mg% respectively.

As shown in Fig. 1, stock solutions of antibiotic were diluted from a 1st to 11th screw cap tube, to obtain two-fold dilutions. Each tube had a concentration ten times the final concentration. 18 ml melted Mueller-Hinton chocolate agar(45°C) was added to each tube, the tubes mixed well, and plates poured and allowed to harden. This antibiotic media was kept no longer than 2 weeks in the refrigerator and dried 15 minutes in the incubator before use. The final concentrations of plates were 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.19, 0.09 mcg/ml; the 12th plate serving as a control.

**Inoculum**

A 24 hours. Levinthal’s broth undiluted culture and 0.1 dilution culture were used in this study.

**Inoculation**

Inocula Replication Apparatus was used.

1. The teflon plastic seed plate which contains 36 inoculum reservoirs was sterilized with 70% alcohol for 30 minutes and dried in an incubator.

An aluminum head containing 36 inoculating rods, was autoclaved and dried in a hot air oven.

2. By aseptic technique, 0.5 ml of inoculum for each strain was pipetted into a plastic seed plate reservoir. Inoculating rods were then lowered
into the wells.

3. A piston was then pressed in order to place the inoculating rods on the surface of the media.

4. Lids were replaced, and incubated plates were allowed to dry at room temperature, then inverted and incubated at 37°C in a candle jar for 24 hours.

Results

After 24 hours incubation, readings of the minimal inhibitory concentration were made. An agar plate without antibiotics was used as a positive growth control. The obtainable concentration of antibiotics in blood (24) were compared with M.I.C. and the results are represented in Table II.

All strains tested had the same susceptibility pattern. They were sensitive to ampicillin (M.I.C. equaled 0.09, 1.56 mcg/ml), tetracycline (M.I.C. equaled 0.39 - 1.56 mcg/ml) and chloramphenicol (M.I.C. equaled 0.78-3.125 mcg/ml). All were resistant to streptomycin (M.I.C. equaled 6.25-50 mcg/ml) and sulfadiazine (M.I.C. equaled 25-100 mg %). Thus in this study, 100% of H. influenzae were sensitive to ampicillin, tetracycline and chloramphenicol but resistant to sulfadiazine. With streptomycin, 75% of the strains were in the intermediate range. (Table II)

Fig. II represent the percentage of total isolates for each M.I.C. The comparison of undiluted inoculum and 0.1 dilution, most strains gave the same result, with a maximum difference of a two-fold dilution.

Discussion and Conclusion

Investigation of a susceptibility pattern is based on the comparison of M.I.C. to obtainable blood level of the antibiotic. If M.I.C. is lower than obtainable blood level, this indicates that organism are sensitive to antibiotics; on the other hand, if M.I.C. is higher than obtainable blood level, it indicates that organism are resistant to the antibiotic tested.

According to several investigators, C.S.F. level of antibiotic is lower than blood level (21, 28, 29, 30, 31, 32, 33). Comparison of M.I.C. to blood level and C.S.F. level should be considered in treatment. For therapeutic purposes, increasing the dosage of antibiotic is required to adjust C.S.F. level to equal M.I.C.

Sensitivity patterns of H. influenzae have been studied by others. Hans A. Hirsch and Maxwell Finland (10) recorded the results of a study from the Bacteriological Laboratory of the Boston City Hospital in 1949, 1954, 1959. By the plate dilution method, H. influenzae tested were sensitive to tetracycline, chloramphenicol and streptomycin. Reports from Bristol Laboratory indicated that plate dilution methods, tube dilution methods, and disc methods gave the same results; H. influenzae tested were all sensitive to ampicillin.
In most studies, sensitivity patterns were the same as those we obtained for ampicillin, tetracycline and chloramphenicol. However there were differences in streptomycin and sulfadiazine results.

Alexander (6, 40), Mac Pherson (41) and Pittman (42) have stated that H. influenzae may become resistant to some antibiotics and sulfadiazine because of mutation. When mutation occurred, mechanisms (3) were: prevention of antibiotic diffusion into cells, a change in metabolic pathways forming a new enzyme which inhibited the action of the antibiotic, a change in ribosomal protein structure which produced the new antibiotic resistant enzymes.

ขั้นตอนนี้

การสกัดผลของยับปู้วาและ Sulfadiazine Sod. ที่มีเชื้อ Haemophilus influenzae 19 strains 牢牢เกิดจากการใช้ของ ร.พ.ดนตรีเจ้าหน้าที่ 75 คน โดยเอาเชื้อจากลักษณะผิวน้ำ แยกเชื้อโดยอาศัยคุณสมบัติในการใช้ X และ V factor คือ "satellite phenomenon" ยั้งยืนลายภาพ colony และการชักวิธีวิทำ

จากนั้นจึงน้ำยาทำการทดลองหา sensitivity pattern โดยทดลองกับยา Amoxicillin, Tetracycline, Chloramphenicol, Streptomycin และ Sulfadiazine วิธีที่ใช้คือ Plate dilution ไข่เครื่องมือ เรียนว่า Inocula Replicator การย้ายผลบัว ความเข้มข้นของยาหน้าอยู่ที่สุดที่สามารถย้ายการเจริญของเชื้อได้ภายใน 24 ชั่วโมง เบน M.I.C. โดยมี plate ที่ไม่มีอยู่ใน media เป็น positive control

ผลลัพธ์การ M.I.C. ไปเปรียบเทียบกับการแสดงความในข้อความข้อหน้าในกระดาษที่คือผิวน้ำ ซึ่งได้รับการย้ายใน dose 1 วิริยะ พบว่าเชื้อ sensitive หรือ resist ดังยา จากผลการทดลองพบว่าที่จงหมด susceptibility pattern อย่างไรก็ได้กันคือ

ยา Amoxicillin ค่า M.I.C. 0.09–1.56 mcg/ml

ยา Tetracycline ค่า M.I.C. 0.39–1.56 mcg/ml

ยา Chloramphenicol ค่า M.I.C. 0.78–3.125 mcg/ml

แต่ resist ที่ Streptomycin และ Sulfadiazine Sod.

จากการเปรียบเทียบที่ใช้ undiluted culture, 0.1 dilution culture ปุ่น inoculum ผลปรากฏว่า ค่า M.I.C. ของยาปฏิชีวนะและยาชักพื้นโดยเชื้อที่มีคือ H. influenzae ไม่แตกต่างมาก มีบาง strains เท่านั้นที่ M.I.C. ต่ำกว่านิด 1 dilution.
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<th>Tetracycline mcg/ml.</th>
<th>Chloramphenicol mcg/ml.</th>
<th>Streptomycin mcg/ml.</th>
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Table I  Minimal inhibitory concentration of 19 strains of H. influenzae to 4 antibiotics and sulfadiazine.

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Table II  The sensitivity test of 19 strains of H. influenzae to 4 antibiotics and sulfadiazine.
Fig. I  Anti-biotic two-fold dilution setup
Fig. II  The susceptibility of 19 strains of H. influenzae to 4 antibiotics and sulfadiazone
References


