

Clonal diversity of Haemophilus Influenzae carriage isolated from under the age of 3 years children

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Research note

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Abstract

Objectives: Pharyngeal carriers such as *H. influenzae* seem to constitute the only reservoir and probably the only transmission vehicle of the invasive disease. Due to lack of information about nasopharyngeal isolation rate, epidemiology, antimicrobial resistance and genetic diversity of *H. influenzae* isolate from carries were assigned as main goals. Sampling was carried out as nasopharynx swabs among children less than 6 years old volunteers. After traditional biochemical tests, isolates were confirmed by targeting *omp6* sequence. Following the susceptibility tests, genomic diversity of strains was analyzed by Pulsed-Field Gel Electrophoresis procedure Results: Out of 328 nasopharynx swabs, 73 strains were identified as *H. influenzae*. Susceptibility testing showed that levofloxacin is the most effective antibiotic against and the least effect belonged to tetracycline. Resistance to chloramphenicol (42.2%) and ampicillin (43.3%) was also observed. By genomic analysis of selected *H. influenzae*, 28 PFGE patterns were achieved among which 11 patterns included at least 2 strains. All strains clustered into 25 different clones. The dendrogram analysis of the isolated *H. influenzae* strains showed that some of these strains had a clonal relationship and common genetic origin. According to our results, antibiotic resistance didn't show any significant correlation with the clonality of strains.

Introduction

Haemophilus influenzae as an opportunistic pathogen is responsible for one third of bacterial pneumonia cases among children of 4 months to 4 years old [1]. Most healthy *H. influenzae* pharyngeal carriers are children in south eastern countries who have not vaccinated against of *H. influenzae*. According to reports, the rate of carriage of *H. influenzae* in infants are about 20% and in children 5 to 6 years old are >50% [2]. Incorrectly using antibiotics and excessively prescribing drugs are considered as one the most important factors of resisting to antibiotics. This causes therapeutic failures and continues existence of resistant strains and so further spread of the disease in medical centers [3–7]. *H. influenzae* is a bacterium that resistance to antibiotics such as co-trimoxazole, ampicillin, tetracycline, amoxicillin, cefixime, and ciprofloxacin have been growing and worrying [8–10]. The survival of resistant strains usually results in the fast spread of the resistant types of bacteria and finally the spread of diseases. Therefore, it is very useful to study the trend of clonal spread and trace the origin of strains in epidemiologic studies [11–13]. Among methods used in studying the clonal spread and genetic similarity between the strains of bacteria, we can refer to Pulsed Field Gel Electrophoresis (PFGE), which is used as a golden standard method for all bacteria such as *H. influenzae* [14]. In this method the whole genome of bacteria is digested and the genome patterns resulting from different strains are studied. This technique can show the vertical transmission of resistance genes among the bacteria [15–17]. Studying the pattern of genome can show the genetic similarity between strains susceptible and resistant to antibiotics [14]. No epidemiologic study has been carried out in Iran to determine the spread of *H. influenzae* carriers compared to antibiotics susceptibility of them. Given the studies conducted in different countries, it is necessary to study the pattern of antibiotic resistance and genomic diversion of *H. influenzae* strains. Thus, this study presents exclusive epidemiologic information about *H. influenzae* strains isolated from

children. This can be helpful for choosing an appropriate therapy to remove infections caused by this bacterium. Note also that a high percentage of carriers are children and the nasopharyngeal carriers of *H. influenzae* play an important role in the dissemination of this microorganism in a population. So this study was conducted about healthy children less than 6 years old.

Materials And Methods

Materials and Methods:

Nasopharyngeal samples

Sampling was done within 10 months from June to the late March, 2016. Samples were collected from children less than 6 years old from six medical centers for children including Children's Medical Center and Ameneh, Shobeir, Torkamani and Roghayyeh nurseries. Nasopharynx swabs were collected from 328 volunteers. Samples swabs were immediately put in to stewart transport medium and transferred to lab during 2 to 3 hours. Samples were then cultured in chocolate agar containing 260 µg/ml bacitracin.

Isolation of *H. influenzae* strains

Prior identification of suspected *H. influenzae* colonies was accomplished basing on the scheme utilized in Diagnostic Microbiology Textbook including ureas, indole, motility, oxidase and catalase [18].

Reconfirmation of suspected isolates by PCR

Suspected growth colonies were screened based on the targeting a encoding external specific protein sequence termed to *ompP6*. This sequence as conserved sequences were determined in both encapsulated and non-encapsulated *H. influenzae* [19]. In short. Crude DNA of suspected isolates directly was extracted taking advantage of a commercial high pure extraction kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufactures recommendation. Quantity of extracted DNA was measured by NanoDrop ND-1000® Spectrophotometer (NanoDrop, Wilmington, DE, USA) and adjusted to 500 ng µL⁻¹. Amplification was done on Gene Amp PCR system (Applied Biosystem, USA) by uniplex PCR method in total volume 25 µl containing; 12 µl master amplicon (Biolab, New England, UK), 1 pmol of each forward and reverse primers, a 1 µl of crude DNA as the template and 10 µl distilled water. Using program an initial cycle of denaturation 94°C for 3 minutes, followed by 25 cycles of 94 minute, for 1min, 60°C for 1min and 72°C for 1 min with a terminal extension for 10min. Resulting PCR were visualized with 1% agarose gel (KBC, Max Pure agarose, Spain). *H. influenzae* ATCC 9007 and *Pseudomonas aeruginosa* ATCC 27853 were served as positive and negative strains. (Additional file 1).

Assessing Antibiotic Sensitivity

Susceptibility testing was done based on the CLSI recommended guidelines [3] using specific antibiotics disks (BD BBLTM Sensi Disc™) include Levofloxacin 5µg, Ciprofloxacin 5µg, Ceftriaxone 30µg, Cefotaxime 30µg, Co-trimoxazole 23/75µg, Chloramphenicol 30µg, Ampicillin 10µg, and Tetracycline 30µg on the Hemophilus test medium (HTM) containing Muller hinton agar supplemented by 15µg/ml NAD and 5µg bovine hematin considering Clinical Laboratory Standards Institute. (Fig. 1). Strains resistant to ampicillin and chloramphenicol and the strain showed intermediate resistance were selected to determine the Minimal Inhibitory Concentration (MIC) of ampicillin and chloramphenicol. Microdilution Broth was used to determine MIC [3]. *H. influenzae* ATCC 49247 was used as the standard control strain.

Studying Molecular Epidemiology

For studying molecular epidemiology of the collected strains, Pulsed Field Gel Electrophoresis (PFGE) was used. The protocol was described previously by Saito *et al.* [14]. Briefly, the plugs were digested with *Sma*I (30U) within 6 hours under 25°C. The program of CHEF-DR III apparatus (Bio-Rad, Richmond, Calif.) was adjusted for the movement of digested bands as the pulse time of 0.5 to 10 s for 16 hours and 10 to 17 s for four hours under the temperature of 4 °C, the voltage of 6 V/cm² and the angle of 120°. The analysis of bands was performed by GelCompare II software (Applied Maths, Belgium). *Salmonella branderup* H9812 was used as size marker.

Results

Identifying and Confirming Samples Containing *H. influenzae*

In this research, 328 collected swabs were evaluated based on biochemical tests in which 108 samples (32.93%) were positive for *H. influenzae*. consequently, considering *omp6* targeted sequence, 73 (22.25%) strains precisely reconfirmed. Mentioned strains were adjusted to susceptibility testing and clonal diversity assessment as well.

Antibiogram and MIC

According to CLSI instructions, the resistance pattern of confirmed strains to selected antibiotics was assessed and reported based on the percentage of resistant, intermediate and susceptible strains (Fig. 1). As observed in Fig. 1, the most effective antibiotics were levofloxacin 100%, ciprofloxacin 92.2%, ceftriaxone 80%, cefotaxime 78.8%, co-trimoxazole 37.8%, chloramphenicol 33.4%, ampicillin 25.6%, and tetracycline 6.7% respectively. Accordingly, the most effective drug against these strains was levofloxacin

and the least effect belonged to tetracycline. MIC of resistant and intermediate analysed strains for two assessed antibiotics (ampicillin and chloramphenicol) was checked followed by Kirby- Bauer break point determination as well. (Additional file 2). Among 57 strains for which the MIC of ampicillin was studied, 20 strains were identified by disk diffusion as intermediate and 37 were resistant. Among them 38 strains were susceptible by MIC. Among 49 strains which the MIC of chloramphenicol was studied for them, using disk diffusion method, 20 strains were identified as intermediate and 29 were resistant. According to MIC, 47 strains were susceptible by MIC. Considering the susceptibility pattern via disc diffusion procedure all isolates were determined as multidrug resistant (MDR) strains. Detailed data listed in Table 1.

Genomic Model of Nasopharynx Strains by PFGE

For PFGE analysis, 45 susceptible and resistant strains were randomly selected among nasopharynx strains. Considering a 90 percent similarity, 28 PFGE patterns were achieved among which 11 patterns included at least 2 strains. The strains clustered into 25 different clones. Clone A had 5 identical strains (strains No. 130, 24, 36, 38, and 48). Clone B had 4 identical strains (strains No.120, 131, 47, and 55). Clone C, D and E contains 3 strains. And finally, clones F, G, H, I, J and K included two strains. Each of the remaining strains formed a separate clone (Fig. 2).

Discussion

H. influenzae is one of the most important reason of pneumonia and meningitidis in children under 6 years old and the sixth leading cause of death in developing countries, highlighting on the importance of this bacterium in medicine [20, 21]. According to various reports about the increase of the resistance of *H.influenzae* to antibiotics, it is particularly important to do cross-sectional studies on the resistance of this bacterium in terms of selecting appropriate antibiotic and avoiding inappropriate ones. In treating the patients the antibiotic resistance pattern of this bacterium has been widely studied in recent years. In 2010, Bae et al. reported the percentage of resistance as 58.5%, 23.3%, 18.7%, 17%, 10.4% and 81% relative to ampicillin, ceforoxim, clarithromycin, cefaclor, amoxicillin, and chloramphenicol respectively [22]. However, levofloxacin and cefotaxime had the strongest effect in this study. Within 20 years, Ladhani et al identified 6805 strains of *H. influenzae* from CSF. More than half of them (54.9%) were type B. Resistance to ampicillin, trimethoprim, tetracycline, and chloramphenicol were 16.2%, 7.7%, 1.8%, and 1.2% respectively. All strains were susceptible to cefotaxime and only 0.06% was resistant to rifampin. Based on the results of them research, rifampicin and cefotaxime have the most effect on *H. influenzae* isolates. This shows the changes on antibiotic susceptibility among the strains of *H. influenzae* [23]. In Iran the results of susceptibility of *H. influenza* showed 85% of the isolates were susceptible to chloramphenicol and 71% to ampicillin and cefotaxime [24]. In our study, 90 percent of strains were susceptible to tetracycline. Compare to other countries, this level has been increased. The resistance to chloramphenicol and ampicillin were 42.2% and 43.3%, respectively which is similar to other countries. In terms of co-trimoxazole, 57.8% of strains were resistant. This finding is similar to Malaysia [25].

On the other hand, due to the pathogenic importance, *H. influenzae* has widely considered in epidemiologic studies on genetic similarities [19]. Given the dendrogram of the studies strains (Fig. 2), it is observed that a number of strains categorized in one clone (e.g. clone A and B). As the studied population was children under 6 years old from different centers, it is concluded that these strains circulated among children. The strains of clone A, isolated from five different children, are identical in terms of resistance to antibiotics except chloramphenicol. As the same the strains of clone B have identical resistance, except for chloramphenicol which is different in one strain. Regardless of intermediate resistance, the strains of clones D, E, F, G, and H have the same resistance pattern. The strains of clones I and J are partially different in their resistance. (Fig. 2).

The resistance pattern of one strain in clone B is identical to other strains of that clone except chloramphenicol. In terms of clone C, the strain (strain No. 89) that closely related to other strains, is different from them in resistance pattern. This may be because of the difference in the genetic algorithm. In the strains forming clone A and B, susceptibility to ceftriaxone, levofloxacin, ciprofloxacin and cefotaxime was observed. This showed that these antibiotics have a good therapeutic effect on these strains.

As the studied strains showed a less percentage of resistance to these antibiotics, these drugs can be a good choice for Haemophilus-related infections. Considering chloramphenicol and ampicillin resistant samples, the strains resistant to ampicillin are more identical in terms of genetic similarities and showed more clones comparing with strains resistant to chloramphenicol. This may mean that strains resistant to ampicillin are more resistant to common treatment than strains resistant to chloramphenicol. As seen in Figure 1 despite different antibiotic resistance pattern of ampicillin resistant isolates, these strains clustered in a similar clone. This also applies in the case of strains resistant and susceptible to chloramphenicol.

Limitations

The dendrogram analysis of Haemophilus strains showed that some of these strains had clonal relations and common genetic origin. It was also observed that strains resistant and susceptible to ampicillin and chloramphenicol can be placed in the same or different clones. In fact, the patterns of antibiotic resistance did not show a significant correlation with the clonality of the strains. Chloramphenicol resistance transferring in these strains may be due to horizontal transmission. It seems that more research is needed to find the relationship between clonality and resistance of bacteria to antibiotics.

Declarations

Ethics approval and consent to participate: This study was approved by Pasteur institute of Iran ethics committee All performed on the enlarged ethical statement B 9008 meeting number in Pasteur institute of Iran. In this study, all ethics including Ethics and Consent to participate from their parents have been collected in the research.

Consent to publication: Not applicable

Availability of data and material: All the results of this study have been classified and maintained by the dissertation in the Pasteur Institute of Iran. We have indeed provided all raw data on which our study is based. *Competing Interests:* The authors declare that they have no competing interests.

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Authors' contributions: All authors read and approved the manuscript. Contributions of the authors in this study was as follow:

F S: Perform laboratory tests performing.

B M: Designing the study, interpretation of results and writing the manuscript.

S F M: Designing, Interpretation of Results, Proof reading.

F H: Performing laboratory tests.

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Conflict of interest

The authors disclose that there is no conflict of interest.

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References

1. Brook I, Hausfeld JN: *Recovery of interfering bacteria in the nasopharynx following antimicrobial therapy of acute maxillary sinusitis with telithromycin or amoxicillin-clavulanate. Antimicrobial agents and chemotherapy* 2005, 49(11):4793–4794.

2. Mukundan D, Ecevit Z, Patel M, Marrs CF, Gilsdorf JR: *Pharyngeal Colonization Dynamics of Haemophilus influenzae and Haemophilus haemolyticus in Healthy Adult Carriers. Journal of clinical microbiology* 2008, 46(4):1575.
3. Moghaddam MM, Aghamollaei H, Kooshki H, Barjini KA, Mirnejad R, Choopani A: *The development of antimicrobial peptides as an approach to prevention of antibiotic resistance. Reviews in Medical Microbiology* 2015, 26(3):98–110.
4. Moghaddam MM, Barjini KA, Ramandi MF, Amani J: *Investigation of the antibacterial activity of a short cationic peptide against multidrug-resistant Klebsiella pneumoniae and Salmonella typhimurium strains and its cytotoxicity on eukaryotic cells. World Journal of Microbiology and Biotechnology* 2014, 30(5):1533–1540.
5. Andersson DI, Hughes D: *Antibiotic resistance and its cost: is it possible to reverse resistance? Nature Reviews Microbiology* 2010, 8(4):260.
6. Levy SB, Marshall B: *Antibacterial resistance worldwide: causes, challenges and responses. Nature medicine* 2004, 10(12s):S122.
7. Moghaddam MM, Abolhassani F, Babavalian H, Mirnejad R, Barjini KA, Amani J: *Comparison of in vitro antibacterial activities of two cationic peptides CM15 and CM11 against five pathogenic bacteria: Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio cholerae, Acinetobacter baumannii, and Escherichia coli. Probiotics and antimicrobial proteins* 2012, 4(2):133–139.
8. Tristram S, Jacobs MR, Appelbaum PC: *Antimicrobial resistance in Haemophilus influenzae. Clinical microbiology reviews* 2007, 20(2):368–389.
9. Mojgani N, Rahbar M, Taqizadeh M, Ashtiani MP, Mohammadzadeh M: *Biotyping, capsular typing, and antibiotic resistance pattern of Haemophilus influenzae strains in Iran. Japanese journal of infectious diseases* 2011, 64(1):66–68.
10. Hasegawa K, Chiba N, Kobayashi R, Murayama SY, Iwata S, Sunakawa K, Ubukata K: *Rapidly increasing prevalence of β -lactamase-nonproducing, ampicillin-resistant Haemophilus influenzae type b in patients with meningitis. Antimicrobial agents and chemotherapy* 2004, 48(5):1509–1514.
11. Gazagne L, Delmas C, Bingen E, Dabernat H: *Molecular epidemiology of ampicillin-resistant non- β -lactamase-producing Haemophilus influenzae. Journal of clinical microbiology* 1998, 36(12):3629–3635.
12. Cohen ML: *Epidemiology of drug resistance: implications for a post-antimicrobial era. Science* 1992, 257(5073):1050–1055.
13. Nazir J, Urban C, Mariano N, Burns J, Tommasulo B, Rosenberg C, Segal-Maurer S, Rahal JJ: *Quinolone-resistant Haemophilus influenzae in a long-term care facility: clinical and molecular epidemiology. Clinical infectious diseases* 2004, 38(11):1564–1569.

- 14.Saito M, Umeda A, Yoshida S-i: *Subtyping of Haemophilus influenzae Strains by Pulsed-Field Gel Electrophoresis. Journal of clinical microbiology* 1999, *37(7)*:2142–2147.
- 15.Yano H, Suetake M, Kuga A, Irinoda K, Okamoto R, Kobayashi T, Inoue M: *Pulsed-field gel electrophoresis analysis of nasopharyngeal flora in children attending a day care center. Journal of clinical microbiology* 2000, *38(2)*:625–629.
- 16.Herrero A, Rodicio M, González-Hevia M, Mendoza M: *Molecular epidemiology of emergent multidrug-resistant Salmonella enterica serotype Typhimurium strains carrying the virulence resistance plasmid pUO-StVR2. Journal of Antimicrobial Chemotherapy* 2005, *57(1)*:39–45.
- 17.Herschleb J, Ananiev G, Schwartz DC: *Pulsed-field gel electrophoresis. Nature protocols* 2007, *2(3)*:677.
- 18.Winn WC: *Koneman's color atlas and textbook of diagnostic microbiology*: Lippincott williams & wilkins; 2006.
- 19.Weinberg G, Towler D, Munson R: *Lipoproteins of Haemophilus influenzae type b. Journal of bacteriology* 1988, *170(9)*:4161–4164.
- 20.Azzari C, Moriondo M, Indolfi G, Massai C, Becciolini L, de Martino M, Resti M: *Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by Streptococcus pneumoniae in Italian children. Journal of medical microbiology* 2008, *57(Pt 10)*:1205.
- 21.Rudan I, Boschi-Pinto C, Biloglav Z, Mulholland K, Campbell H: *Epidemiology and etiology of childhood pneumonia. Bulletin of the world health organization* 2008, *86*:408–416B.
- 22.Bae S, Lee J, Lee J, Kim E, Lee S, Yu J, Kang Y: *Antimicrobial resistance in Haemophilus influenzae respiratory tract isolates in Korea: results of a nationwide acute respiratory infections surveillance. Antimicrobial agents and chemotherapy* 2010, *54(1)*:65–71.
- 23.Ladhani S, Heath PT, Ramsay ME, Slack MP: *Changes in antibiotic resistance rates of invasive Haemophilus influenzae isolates in England and Wales over the last 20 years. Journal of antimicrobial chemotherapy* 2008, *62(4)*:776–779.
- 24.FARAJZADEH SA, Mosavy N, Tavacol H: *ISOLATION AND ANTIBIOGRAM PATTERN OF HAEMOPHILUS INFLUENZAE ISOLATED FROM BRONCHIAL WASHING OF PATIENTS UNDERGOING BRONCHOSCOPY. 2004.*
- 25.Mohd-Zain Z, Kamsani N, Ismail I, Ahmad N: *Antibiotic susceptibility profile of Haemophilus influenzae and transfer of co-trimoxazole resistance determinants. Trop Biomed* 2012, *29*:372–380.

Table

Source of collected samples	Number of specimen's	Multi Drug Resistant isolates				
		Two ABs	Three ABs	Four ABs	Five ABs	Six ABs
Children's Medical Center	15(20.55%)	8(10.96%)	3(4.11%)	3(4.11%)	1(1.37%)	-
Ameneh Nursery	15(20.55%)	8(10.96%)	3(4.11%)	3(4.11%)	-	1(1.37%)
Shobeir Nursery	26(35.61%)	5(6.85%)	8(10.96%)	9(12.32%)	2(2.74%)	2(2.74%)
Torkamani Nursery	9(12.33%)	5(6.85%)	3(4.11%)	-	-	1(1.37%)
Roghayyeh Nursery	8(10.96%)	4(5.48%)	2(2.74%)	2(2.74%)	-	-
Total (%)	73 (100%)	30(41.1%)	19(26.03%)	17(23.29%)	3(4.11%)	4(5.48%)

ABs, Antibiotics.

Figures

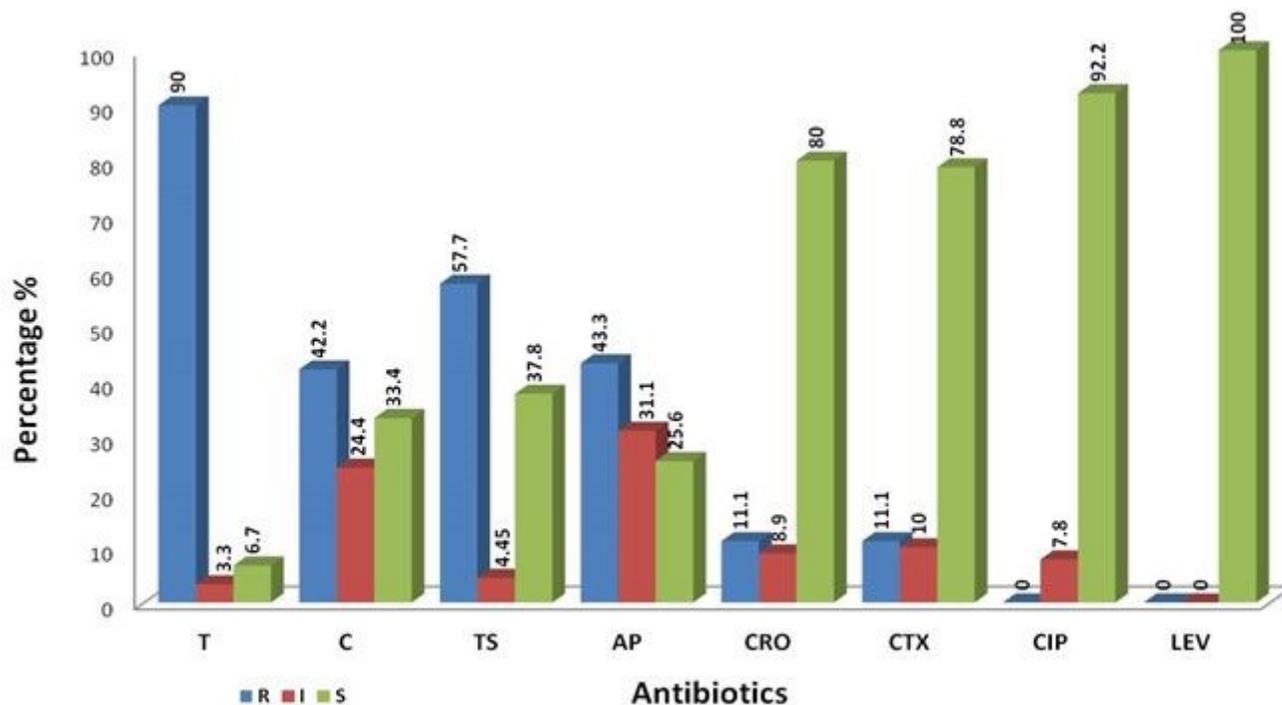


Figure 1

Susceptibility patterns of nasopharyngeal isolates basing on the Minimum Bactericidal Concentration criteria. T, Tetracycline; C, Chloramphenicol; TS, Co-trimoxazole; AP, Ampicillin; CRO, Ceftriaxone ;CTX, Cefotaxime; CIP, Ciprofloxacin; LEV, Levofloxacin.

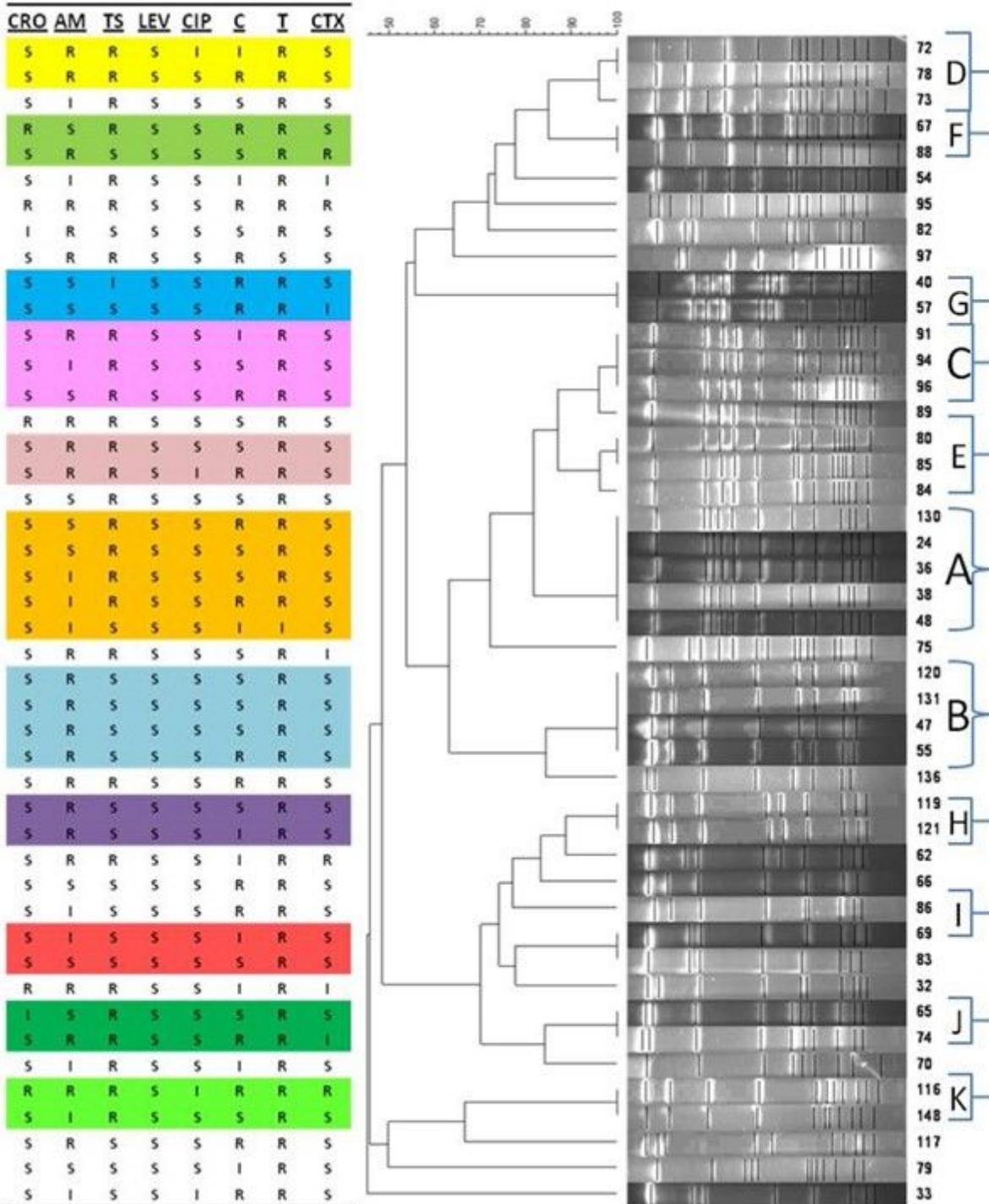


Figure 2

Genomic patterns of nasopharyngeal H. influenza isolates by PFGE. Randomly analysed strains PFGE pattern was assessed by a CHEF-DR III software.

Supplementary Files

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