Anti-Haemophilus influenzae activity of Pseudomonas aeruginosa

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ABSTRACT: Interspecies interactions can cause false-negative culture results for microorganism the growth of which is inhibited by the other one in mixed infections. In this study it was aimed to show whether there was an interaction between Haemophilus influenzae and Pseudomonas aeruginosa. We used five different P.aeruginosa isolates and five H.influenzae isolates. We suspended all isolates and we inoculated H.influenzae suspensions first onto chocolate agar media. Then, we inoculated P.aeruginosa suspensions onto Haemophilus-inoculated media as a line in 1 cm-width. We also tested the interactions in broth media. We cross-inoculated each H.influenzae suspensions with each P.aeruginosa suspensions. On solid media, all P. aeruginosa isolates inhibited the growth of H. influenzae colonies which were observed to be inhibited within approximately 1 mm close to P.aeruginosa inoculation lines. No H.influenzae growth was also observed from the cross-inoculation wells except P.aeruginosa colonies. Both solid and broth media tests revealed complete anti-Haemophilus activity of P.aeruginosa isolates. In conclusion, we demonstrated that P.aeruginosa inhibited the growth of H.influenzae in laboratory conditions. We concluded that clinicians should not overlook H.influenzae in cultures which reveal P.aeruginosa dominantly in suspicious clinical specimens.

Keywords: Anti-haemophilus, Haemophilus influenzae, Pseudomonas

INTRODUCTION

Haemophilus influenzae is a Gram-negative coccobacillus which can cause various infectious diseases such as pneumonia, meningitis, acute otitis media, acute sinusitis, and acute exacerbations of chronic bronchitis (Killian, 2003; Tristram et al., 2007; Harrison et al., 2012; Ruohola et al., 2006). Particularly in upper respiratory tract infections, H. influenzae can be isolated from cultures of appropriately collected clinical specimens such as sputum, bronchoalveolar lavage, and endotracheal aspirate (Tristram et al., 2007; Brusselaers et al., 2012). H. influenzae needs factor X (Hemin) and factor V (Nicotinamide adenine dinucleotide-NAD) to grow on culture medium (Killian, 2003). It can grow both on chocolate agar as this medium contains factors X and V, and on 5% sheep blood agar as tiny colonies can be examined only around beta-hemolytic Staphylococcus aureus colonies which is called “satellite phenomenon” (Killian, 2003).

Pseudomonas aeruginosa is a Gram-negative rod which can cause serious infections that lead to morbidity and mortality (Brusselaers et al., Jefferies et al., 2012). P. aeruginosa commonly cause nosocomial outbreaks, especially in immunsuppressed and long-time-hospitalized or intensive care unit patients (Brusselaers et al., Jefferies et al., 2012). It can be easily isolated from cultures of clinical specimens and it can grow on most culture media (Brusselaers et al., Jefferies et al., 2012).

Interspecies interactions are important for survival of microorganisms and ecological balance (Hockey et al., 1982; Tano et al., 1999). This relationship is important if it can cause false-negative culture results for inhibited microorganism and this may lead clinician to give insufficient treatment to the patient. Interaction between P. aeruginosa and Candida albicans is better explained in previous studies (Hockey et al., 1982; Keceli Özcan et al., 2012; Kerr et al., 1994; Kerr et
al., 1999; Grillot et al., 1994; Kaleli et al., 2007; Morales et al., 2010; Holjombe et al., 2010; Gibson et al., 2009; Nseir et al., 2007). It has been noted that *P. aeruginosa* has anti-candidal activity (Keceli Özcan et al., 2012; Kerr et al., 1994; Kerr et al., 1999; Grillot et al., 1994; Kaleli et al., 2007; Morales et al., 2010; Holjombe et al., 2010; Gibson et al., 2009; Nseir et al., 2007). But rare performed studies focused on investigating an interaction between *P. aeruginosa* and *H. influenzae* (van Belkum et al., 2000; Ojano-dirain et al., 2011).

In this study it aimed to show whether there was an interaction between *H. influenzae* and *P. aeruginosa* both on solid and broth culture media.

**MATERIALS AND METHOD**

**Isolates**

We used five different *P. aeruginosa* isolates and five *H. influenzae* isolates all of which were obtained from sputum specimens of different patients. As we do in routine culture, we inoculated sputum specimens onto 5% blood agar, eosin methylene blue agar and chocolate agar media. After 24 h incubation in 35-37º C in 5-10% CO₂ incubator we examined the culture media. For *P. aeruginosa*, we identified suspicious predominant or dominant colonies either with conventional methods as microscopic examination, oxidase test and biochemical tests such as triple sugar iron or with automated identification systems as VITEK (bioMérieux, France) or BD Phoenix (Becton Dickinson, USA). For *H. influenzae*, we gave special attention to colonies which either grew predominantly on chocolate agar or did not grow on blood agar or grew as tiny colonies around other beta-hemolytic colonies. For Gram-negative coccobacillus colonies, we used API NH (bioMérieux, France) semi-automated identification kit. After full identification of each microorganism, we stored the isolates in glycerin in -20º C until performing the tests of this study.

**Interaction tests**

We subcultured *P. aeruginosa* isolates on blood agar and *H. influenzae* isolates on chocolate agar. After 24 h incubation in 35-37º C in 5-10% CO₂ incubator, we performed the interaction tests. We suspended all of the isolates using 0.5 McFarland Standard densities. First, we inoculated *H. influenzae* suspensions on five different chocolate agar media with sterile swabs. Then, we inoculated *P. aeruginosa* suspensions on *Haemophilus*-inoculated media as a line in 1 cm-width with swabbing once in duplicates. As a result, on five chocolate agar media, a total of 10 symmetrical inoculations of *Pseudomonas* on *Haemophilus* were obtained. We re-incubated the media in the same conditions.

We also used sterile 96-well-microplates to test the interactions in broth media. We used tryptic soy broth (TSB) (Oxoid, England). We distributed 90 µL of TSB into the wells of plates. We diluted the bacterial suspensions of 0.5 McFarland in 1:10 folds to get a final concentration of 10⁶ CFU/mL. Then we inoculated 10 µL of each suspension into separate wells as a concentration of 10⁵ CFU/100 µL-well. And we distributed the *Pseudomonas* suspensions in a different microplate, and then we added the *Haemophilus* suspensions into the wells containing *Pseudomonas* using 10 µL of each. As a result we obtained five *H. influenzae* wells, five *P. aeruginosa* wells, and 25 cross-inoculation wells. We incubated the plate in the same condition of solid culture media (Figure 1).

After incubation, 1:10 folds serial dilutions were made in eight serial wells to make the inoculation results countable. 10 µL of each diluted well of the broth cultures were taken and inoculated onto chocolate agar media in duplicates and re-incubated in the same conditions to assess the interaction quantitatively.

After all the culture examinations, we observed them in the second and the third days whether there are any changes about the interactions.

We used chocolate agar for the isolate as the growth controls, and used single-inoculated control wells for each isolate in the microplate (Figure 1).

**Culture examination**

After 24 h incubation, culture media were examined for interactions by two microbiologists experienced for at least two years. The media were examined whether there were any inhibition in isolates or there were any interactions. For solid media tests, any inhibition of growth of *H. influenzae* near the inoculation line of *Pseudomonas* was determined as “Anti-*Haemophilus* activity” of *P. aeruginosa*. For broth media, any quantitative decreases in number of colony-forming unit of *H. influenza* which was re-inoculated onto solid media were determined as “Anti-*Haemophilus* activity”. For this evaluation, the results of the cross-incubation were compared to pure inoculations (Positive controls) of the isolates.
RESULTS

Broth media

The inoculation of the wells that single-inoculated suspensions (Positive controls) of isolates revealed growth, but no *H. influenzae* growth was observed from any of the cross-inoculation wells. From these wells, only *P. aeruginosa* colonies were observed, so broth media tests revealed full anti-*Haemophilus* activity of *P. aeruginosa* isolates (Figure 1).

Solid media

On five chocolate agar media, all *P. aeruginosa* isolates inhibited the growth of *H. influenzae* colonies which were observed to be inhibited within approximately 1 mm close to *P. aeruginosa* inoculation lines. Solid media tests revealed that there is anti-*Haemophilus* activity of *P. aeruginosa* (Figure 2).

DISCUSSION

Interspecies interactions can cause false-negative culture results for microorganism the growth of which is inhibited by the other one for mixed infections (van Belkum et al., 2000; Ojano-dirain et al., 2011). This situation may cause *H. influenzae* to be overlooked by both microbiologists and clinicians of the patient, and this may lead to insufficient treatment (Harrison et al., 2012; Jefferies et al., 2012; Keceli Özcan et al., 2012).

In our study we have found that there is an in vitro interaction between *P. aeruginosa* and *H. influenzae*. *P. aeruginosa* inhibited the growth of *H. influenzae* on both of solid and broth media.

In this study we tested the bacterial interaction in both of solid and broth culture media. We aimed to find out if there were interactions in fluid and solid conditions and by this way, our findings would demonstrate the absolute interaction which does not vary due to in vitro differences.

In our study we chose the isolates from sputum specimens. Because the probable interaction result between these two microorganisms is mostly found in upper respiratory tract specimens as either asymptotically or infectious disease, so theoretically they can be found in sputum together (Brusselaers et al., 2012; van Belkum et al., 2000; Ojano-dirain et al., 2011). Therefore our findings after the test would be more significant for clinical perspectives.

Van Belkum et al. (van Belkum et al., 2000) reported that three of five sputum specimens revealed negative for *H.
influenzae but positive for *P. aeruginosa* though these specimens were positive for each microorganism by PCR. They reported a similar result for *Staphylococcus aureus* negativity in *Pseudomonas*-revealed cultures. This report supports our result of interaction and our consideration about overlooking of *H. influenzae*.

Ojano-Dirain et al. (Ojano-dirain et al., 2011) studied to demonstrate whether *H. influenzae* promotes biofilm formation of *P. aeruginosa* in posttympanostomy tube otorrhea but they reported that they couldn’t find any positive effects of *Haemophilus* on this topic. They claim that polymerase chain reaction (PCR) method showed *H. influenzae* and *P. aeruginosa* commonly coexisted on explanted tympanostomy tubes and ossicular prosthesis. So they also claim that the use of high-dose ototopical therapy to clear *H. influenzae* infection reduced subsequent *P. aeruginosa* biofilm formation according to their unpublished observations. This claim supports that *P. aeruginosa* and *H. influenzae* coexists in patient and in clinical specimen but *P. aeruginosa* inhibits the growth of *Haemophilus*; so clinicians should take more attention so as not to overlook *Haemophilus* in cultures which reveal dominant *Pseudomonas* in *Haemophilus*-suspicious infections (Van Belkum et al., 2000; Ojano-dirain et al., 2011).

There have been so many studies focused on antifungal activity of *P. aeruginosa* isolates which demonstrated that between 2-52% of *P. aeruginosa* have this kind of activity (Hockey et al., 1982; Keceli Özcan et al., 2012; Kerr et al., 1994; Kerr et al., 1999; Grillot et al., 1994; Kaleli et al., 2007; Morales et al., 2010; Holjombe et al., 2010; Gibson et al., 2009; Nseir et al., 2007).

(Kaleli et al., 2007) showed in vitro antifungal activity of *P. aeruginosa* in a range of between 25-48% of the isolates. They reported that *Pseudomonas* showed complete inhibition on *Candida* in subcutaneous experiment in rats. Besides, in routine we observe that particularly in intensive care unit patients, respiratory tract specimen cultures reveal both *P. aeruginosa* and *Candida* colonies on the same media (Unpublished data); so we consider that more clinical trials have to be done to prove that *Pseudomonas* always and completely inhibit the growth of *Candida* in vivo or in clinical
specimens. In the same way, we do not know whether *P. aeruginosa* completely inhibits *H. influenzae* in vivo or in clinical specimens just relying on our results because of insufficient data of previous clinical trials.

Keceli Özcan et al. (9) found that *P. aeruginosa* showed anti-*Candidal* activity in solid media in rates of between 62-73% of isolates, and they also demonstrated that *Pseudomonas* and *Candida* isolates inhibit biofilm formation of each other. They considered this was probably caused by signal mediated interaction. This antifungal effect was considered to be dependent either on redox-active pyocyanin, bacteriosin, phenazine or biofilm formation of *Pseudomonas* (Keceli Özcan et al., 2012; Kerr et al., 1994; Kerr et al., 1999; Morales et al., 2010; Holjombe et al., 2010; Gibson et al., 2009).

We do not know whether these factors cause the interaction found in our study. In this study we didn’t perform any molecular tests because those tests such as PCR is not routinely performed in every laboratories and if only culture from upper respiratory tract specimens are processed, the result as *P. aeruginosa* always seem to be the most important clinical situation of the patient to be recovered, so *H. influenzae* is usually overlooked or not taken serious or important by clinician (Brusselaers et al., 2012; Van Belkum et al., 2000; Ojano-dirain et al., 2011).

**CONCLUSION**

In conclusion, we demonstrated that *P. aeruginosa* inhibited growth of *H. influenzae* in laboratory conditions. Besides, we do not consider that this interaction occurs the same in vivo while this consideration should be proved by PCR or by in vivo clinical trials. In addition, after eradication of *Pseudomonas* from the patient, control cultures can be done to clarify if *Haemophilus* still exists in the clinical specimen. As a result, we conclude that clinicians should not overlook *H. influenzae* in cultures which reveal *P. aeruginosa* dominantly in suspicious clinical specimens.

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