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REVIEW ARTICLE

# Diagnosis of biofilm infections in cystic fibrosis patients

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Chronic *Pseudomonas aeruginosa* biofilm lung infection in cystic fibrosis patients is the best described biofilm infection in medicine. The initial focus can be the paranasal sinuses and then follows repeated colonization and infection of the lungs by aspiration. The matrix of the biofilms is dominated by alginate and the pathogenesis of tissue damage is immune complex-mediated chronic inflammation dominated by polymorphonuclear leukocytes and their products (DNA, oxygen radicals and proteases). The *P. aeruginosa* biofilm infection can be diagnosed by microscopy of lung tissue, sputum and mucus from the paranasal sinuses, where aggregates of the bacteria are found surrounded by the abundant alginate matrix. Specific PNA-FISH probes can be used to identify *P. aeruginosa* and other pathogens *in situ* in the biofilms. Growth of mucoid colonies from the locations mentioned above is also diagnostic for biofilm infection. Rise of specific anti-*P. aeruginosa* antibodies is likewise diagnostic, IgG in serum in case of lung infection, sIgA in saliva or nasal secretions in case of paranasal sinus infection. Similar approaches have been developed to diagnose chronic biofilm infections in cystic fibrosis caused by other pathogens e.g., *Stenotrophomonas, Burkholderia multivorans, Achromobacter xylosoxidans* and *Mycobacterium abscessus* complex.

Key words: Biofilms; microbial biofilms; biofilm infection; *Pseudomonas aeruginosa*; cystic fibrosis.

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Bacteria and fungi have two different life styles. The planktonic life style is characterized by individual cells or cells in small chains (e.g., streptococci) or small clusters (e.g., staphylococci) which float or swim around unprotected from toxic substances, bacteriophages or phagocytosing cells. It is, therefore, a dangerous life style (1). The biofilm life style, on the other hand, is characterized by aggregates of cells surrounded by a self-produced matrix which may also contain components from the surrounding environment e.g., the host (1). Such microbial aggregates may be adhering to a natural or artificial surface (sessile growth, *adhering biofilms*) e.g., teeth, epidermis cells, intravenous lines or artificial joints or they may be located in the tissue (*non-adhering biofilms*) e.g., in mucus on mucosal membranes, sputum, or inside chronic wounds. Importantly, biofilm growing bacteria are much more tolerant to antibiotics and to the body's innate and adaptive defense mechanisms. Therefore, biofilm infections are chronic infections and the pathogenesis involves antibody-potentiated chronic inflammation around the biofilms. The inflammation, therefore, is an immune complex-mediated inflammation, as the antibody response contributes to the pathogenesis of infection (2).

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## CYSTIC FIBROSIS AND BIOFILM INFECTION – PATHOGENETIC AND DIAGNOSTIC ASPECTS

Cystic fibrosis (CF) patients suffer from recurrent and chronic sinus and lung infections due to the basic defect of the CFTR protein, which is a

chloride channel (3). This leads to decreased volume of the paraciliary fluid in the airways and impaired mucus detachment (4) which, taken together, interferes with mucociliary transport leading to defective host defense against respiratory bacterial infections. *Pseudomonas aeruginosa*, which causes chronic lung infection in patients with CF, was the first biofilm infection described in humans and is now the most well-studied biofilm infection (Fig. 1A–D) (5). However, *Burkholderia multivorans, Achromobacter xylosoxidans, Stenotrophomonas maltophilia, Dyella species* and *Mycobacterium abscessus* (Fig. 2A–D) (6–8) have also been shown to be able to produce biofilms in CF airways. All of these biofilms are non-adhering in the CF airways.

It has been shown, that the paranasal sinuses often become colonized before the lower airways with *P. aeruginosa,* which are subsequently aspirated (from the post-nasal drip) to the lungs especially during episodes of viral infections of the upper airways (9). *Pseudomonas aeruginosa*, therefore, adapts to the upper airways and forms biofilms from where they repeatedly colonize the lower airways which sooner or later leads to chronic lung infection (10, 11). The mucus of paranasal sinuses is microaerophilic or anaerobic during chronic *P. aeruginosa* sinusitis (12). The lower airways consist of the conductive zone (larynx, bronchi, bronchioles and the terminal bronchioles) where the mucosa is ciliated and where sputum is produced, and the respiratory zone (respiratory bronchioles, alveolar ducts and alveoles), where there is no cilia and no sputum production, and this is where the air exchange takes place (13). During lung infections, polymorphonuclear leukocytes (PMN) are recruited from the capillaries of the involved alveoles and transported to the conductive zone where the abundance of PMNs during phagocytosis of the bacteria mounts a metabolic burst powered by oxygen consumption, formation of reactive oxygen species such as superoxide and, additionally, released PMN-DNA and PMN-proteases (14, 15). The sputum therefore becomes viscid and anaerobic (14). The chronic inflammation around *P. aeruginosa* biofilms is dominated by PMNs in both the respiratory zone and the conductive zone inside sputum (14, 15). In the respiratory zone, this leads to tissue damage and loss of lung function, whereas in the conductive zone it leads to obstruction of the airways (15). The chronic *P. aeruginosa* biofilm lung infection leads to a pronounced IgG antibody

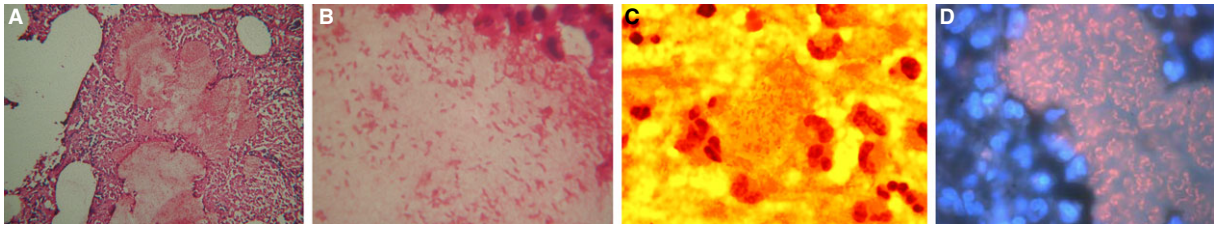


**Fig. 1.** (A) Autopsy (BS242/74) of a CF girl (MLM) who died due to chronic *Pseudomonas aeruginosa* lung infection and 21 precipitating antibodies against *P. aeruginosa* (normal: 0–1). Severely inflamed tissue (pneumonia). Hematoxylin-Eosin stain × 40. (B) Autopsy (BS242/74) of a CF girl (MLM) who died due to chronic *P. aeruginosa* lung infection and 21 precipitating antibodies against *P. aeruginosa*. Gram stain × 1000. (C) Gram-stained sputum from a female CF patient 44 y, an alginate containing biofilm of *P. aeruginosa* is surrounded by polymorphonuclear leukocytes. (D) PNA-FISH staining with a *P. aeruginosa* specific probe of a biofilm in tissue from an explanted lung (15).

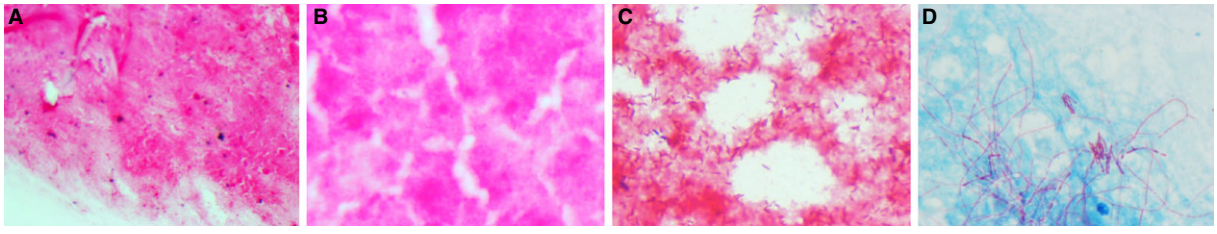


**Fig. 2.** (A) Biofilm of *Achromobacter xylosoxidans* in sputum from a CF patient with chronic lung infection, Gram stain × 1000. (B) Biofilm of *Burkholderia multivorans* in sputum from a CF patient with chronic lung infection, Gram stain × 1000. (C) Biofilm of *Stenotrophomonas maltophilia* in sputum from a CF patient with chronic lung infection, Gram stain × 1000. (D) Biofilm of *Mycobacterium abscessus* in sputum from a CF patient with chronic lung infection. Ziehl-Neelsen stain, × 1000 (8).

response against *P. aeruginosa* components including alginate, and these specific antibodies can be detected in blood and used diagnostically (5, 6, 16–20) (Tables 1–2) (Fig. 3A). Likewise, the antibody response against other biofilm growing CF pathogens can be used diagnostically e.g., *S. maltophilia, B. multivorans, A. xylosoxidans* (Figs 3B, C, D and 4) (21).

**Table 1.** Current laboratory methods for diagnosis *Pseudomonas aeruginosa* and other bacterial biofilms in lung tissue, sputum or mucus from paranasal sinuses in CF

| | |
|---|---|
| Microscopy: | Light microscopy of Gram-stained smears, the biofilms are small aggregates of bacteria (4–100 μm), the matrix is dominated by alginate, and it may take several minutes to find a biofilm. There is abundance of polymorphonuclear leukocytes around the biofilms (Fig. 1A–C, Fig. 2A–D) (15) |
| | FISH microscopy of smears, the biofilms are small aggregates (4–100 μm) and it may take several minutes to find a biofilm (Fig. 1D). There is abundance of polymorphonuclear leukocytes around the biofilms. The signal of the FISH probe is dependent on the number of ribosomes in each bacterial cell and dormant or slow growing bacteria may therefore show weak fluorescence (28) |
| | The polysaccharide matrix of the biofilms can be stained by Alcian blue (30) or Calcofluor (31) |
| Growth of mucoid colonies of *P. aeruginosa* (Fig. 5) (5, 18) | |
| Antibody response: | IgG antibody response in serum to *P. aeruginosa* antigens (proteins, lipopolysaccharide, alginate) (Table 2) (Fig. 3A), (5, 16–19) |
| | sIgA antibody response in saliva or in secretions from the paranasal mucosal membranes to *P. aeruginosa* antigens (proteins, alginate) (22) |
| | In case of other biofilms than *P. aeruginosa*, there is no alginate present and only serum IgG antibodies have been used to diagnose biofilm infection (Fig. 3B–D, Fig. 4) (21, 24) |

**Table 2.** Diagnostic use of three different anti-pseudomonas antibody methods (antibodies in serum) to detect chronic *Pseudomonas aeruginosa* biofilm infection in Scandinavian cystic fibrosis patients (17)

| | Crossed immune-electrophoresis (St-Ag was used) (%) | *Pseudomonas*-CF-IgG Elisa (St-Ag was used) (%) | Exotoxin A Elisa (%) |
|---|---|---|---|
| Specificity | 89 | 83 | 89 |
| Sensitivity | 96 | 97 | 93 |
| Positive predictive value | 87 | 80 | 86 |
| Negative predictive value | 97 | 98 | 95 |
| Positive predictive value | 93 | 85 | 88 |
| Negative predictive value after patients with other Gram-negative infections were excluded | 97 | 97 | 95 |

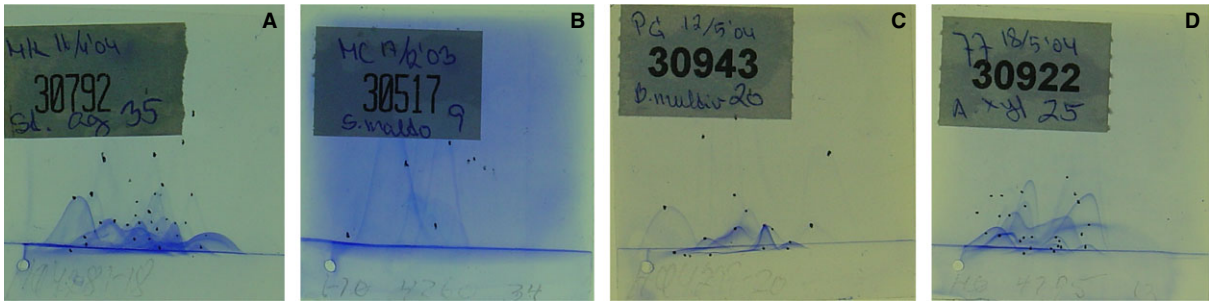


**Fig. 3.** (A) Crossed immunoelectrophoresis of Standard-Antigen (a sonicate of 17 different *Pseudomonas aeruginosa* O-groups) runs against serum from a CF patient with chronic *P. aeruginosa* lung infection. Thirty-five different precipitates are seen (normal 0–1) (5, 29). (B) Crossed immunoelectrophoresis of a sonicate of *Stenotrophomonas maltophilia* antigen runs against serum from a CF patient with chronic *S. maltophilia* lung infection. Nine different precipitates are seen (normal 0–1) (21, 29). (C) Crossed immunoelectrophoresis of a sonicate of *Burkholderia multivorans* antigen runs against serum from a CF patient with chronic *B. multivorans* lung infection. Twenty different precipitates are seen (normal 0–1) (21, 29). (D) Crossed immunoelectrophoresis of a sonicate of *Achromobacter xylosoxidans* antigen runs against serum from a CF patient with chronic *A. xylosoxidans* lung infection. Twenty-five different precipitates are seen (normal 0–1) (21, 29).

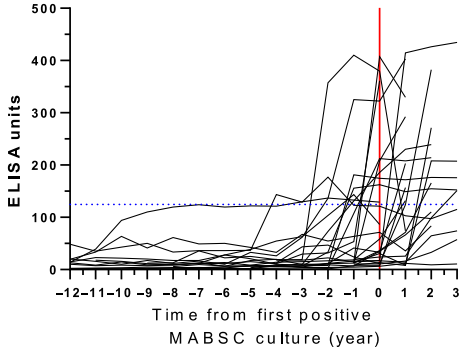


**Fig. 4.** Anti-*Mycobacterium abscessus* complex antibody kinetic in serum from 26 CF patients who developed *M. abscessus* complex pulmonary disease. The dotted horizontal line is the 125 ELISA units (test positive threshold). The dotted vertical line represents the date of first positive culture (reproduced from (20).

The chronic *P. aeruginosa* infection in the paranasal sinuses is also characterized by biofilm formation, but the antibody response is dominated by sIgA against *P. aeruginosa* components including alginate (22, 23). *Pseudomonas aeruginosa* specific sIgA in saliva or in the mucosa of the sinuses can be used diagnostically (Tables 1, 3) (22).

The matrix of *P. aeruginosa* biofilms in CF is dominated by the polysaccharide alginate (polymer of blocks of guluronic- and manuronic acids kept together by $Ca^{++}$) (24) but may also contain other components such as eDNA and LPS from *P. aeruginosa* and components from the host e.g., DNA from PMNs (25). Hyperproduction of alginate is due to mutations in the *mucA* gene which gives rise to mucoid colonies and the frequently simultaneous presence of non-mucoid colonies of the same genotype is due to additional mutations in the *algT* gene (=*algU*) (26). Mucoid biofilms in lung tissue, in sputum and in the paranasal sinuses sputum (Fig. 1A–D) (15, 23), presence of alginate in sputum (27) or growth of mucoid colonies (Fig. 5) can be used diagnostically (18) (Table 1). Importantly, molecular methods based on PCR cannot distinguish between biofilm growing- and planktonically growing bacteria (1).

**Table 3.** Combined diagnostic use of two anti-pseudomonas sIgA measured by ELISA method (antibodies in saliva or nasal mucosa) to discriminate between intermittent lung colonization and no lung colonization/infection with *Pseudomonas aeruginosa* in CF patients. St-Ag and purified alginate was used (ref). Intermittently colonized CF patients are at risk of being chronically infected from a sinus focus with *P. aeruginosa* biofilm infection (22)

| | sIgA in nasal secretions (%) | sIgA in saliva (%) |
|---|---|---|
| Specificity | 81 | 87 |
| Sensitivity | 96 | 72 |
| Positive predictive value | 79 | 87 |
| Negative predictive value | 96 | 52 |

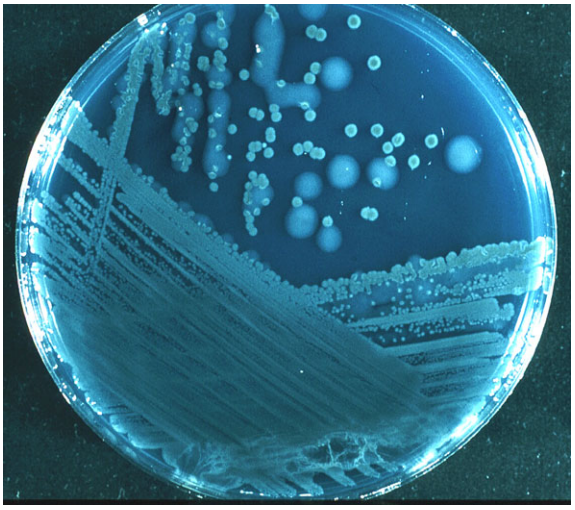

**Fig. 5.** *Pseudomonas aeruginosa* colonies grown from sputum of a CF patient with chronic lung infection. Mucoid (large, slimy) and non-mucoid (small, rough) colonies are seen. The mucoid colonies are diagnostic for biofilm infection (5, 18).

In the future, occurrence of biofilm-specific antigens – like alginate of *P. aeruginosa* – or other molecules should be searched for in other bacterial species. They may then be used for development of chemical methods or development of antibody techniques for diagnosis of biofilm infection. PNA-FISH probes specific for other CF pathogens than *P. aeruginosa* (15) and *S. maltophilia* (28) should also be developed. Automatic microscopy of sputum may be developed using the FISH technique to detect aggregates of bacteria and thereby diagnose biofilm growth in sputum.

## DISCLOSURE

The authors have no issues to disclose.

## REFERENCES

1. Høiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, et al. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect 2015;21(Suppl 1):1–26.
2. Høiby N, Ciofu O, Bjarnsholt T. Pseudomonas aeruginosa biofilms in cystic fibrosis. Future Microbiol 2010;5:1663–74.
3. Boucher RC. New concepts of the pathogenesis of cystic fibrosis lung disease. Eur Resp J 2004;23:146–58.

4. Hoegger MJ, Fisher AJ, McMenimen JD, Ostedgaard LS, Tucker AJ, Awadalla MA, et al. Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis. Science 2014;345:818–22.
5. Høiby N. Pseudomonas aeruginosa Infection in cystic fibrosis. Diagnostic and prognostic significance of Pseudomonas aeruginosa precipitins determined by means of crossed immunoelectrophoresis. A survey. Acta Pathol Microbiol Immunol Scand 1977;262 (Suppl):1–96.
6. Ciofu O, Hansen CR, Høiby N. Respiratory bacterial infections in cystic fibrosis. Curr Opinion Pulmonary Med 2014;19:251–8.
7. Duus LM, Høiby N, Wang M, Schiøtz O, Nørskov-Lauritsen N. Bacteria of the genus Dyella can chronically colonize the airways of patients with cystic fibrosis and elicit a pronounced antibody response. Internat. J Med Microbiol 2013;303:267–9.
8. Qvist T, Eickhardt S, Kragh KN, Andersen CB, Iversen M, Høiby N, et al. Chronic pulmonary disease with Mycobacterium abscessus complex is a biofilm infection. Eur Resp J 2015;46:1823–6.
9. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, et al. Adaptation of Pseudomonas aeruginosa to the airways of cystic fibrosis patients: an evolutionary perspective. Nat Rev 2012;10:841–51.
10. Aasnæs K, Johansen HK, Skov M, Buchwald FF, Hjuler T, Pressler T, et al. Clinical effects of sinus surgery and adjuvant therapy in cystic fibrosis patients – can chronic lung infection be postponed? Rhinology 2000;51:222–30.
11. Ciofu O, Johansen HK, Aasnæs K, Wassermann T, Alhede M, von Buchwald C, et al. aeruginosa in the paranasal sinuses and transplantated lungs have similar adaptive mutations as isolates from the chronically infected CF lungs. J Cystic Fibrosis 2013;12:729–36.
12. Aasnæs K, Rickewelt LF, Johansen HK, von Buchwald C, Pressler T, Høiby N, et al. Decreased mucosal oxygen tension in the maxillary sinuses in patients with cystic fibrosis. J Cystic Fibrosis 2011;10:114–20.
13. Westh JB. Pulmonary Physiology and Pathophysiology. Philadelphia: Lippincott Williams & Wilkins, 2001: 1–162.
14. Kolpen M, Hansen CR, Bjarnsholt T, Moser C, Christensen LD, van Gennip M, et al. Polymorphonuclear leukocytes consume oxygen in sputum from chronic Pseudomonas aeruginosa pneumonia in cystic fibrosis. Thorax 2010;65:57–62.
15. Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol 2009;44:547–58.
16. Pedersen SS, Espersen F, Høiby N, Jensen T. Immunoglobulin A and Immunoglobulin G antibody responses to alginates from Pseudomonas aeruginosa in Cystic Fibrosis. J Clin Microbiol 1990;28:747–55.
17. Pressler T, Karpati F, Granström M, Knudsen PK, Lindblad A, Hjelte L, et al. Diagnostic significance of measurements of specific IgG antibodies to Pseudomonas aeruginosa by three different serological methods. J Cystic Fibrosis 2009;8:37–42.
18. Pressler T, Frederiksen B, Skov M, Garrad P, Koch C, Høiby N. Early rise of anti-Pseudomonas antibodies and a mucoid phenotype of Pseudomonas aeruginosa are risk factors for development of chronic lung infection – a case control study. J Cystic Fibrosis 2006;5:9–15.
19. Mauch RM, Levy CE. Serum antibodies to Pseudomonas aeruginosa in cystic fibrosis: a systematic review. J Cystic Fibrosis 2014;13:499–507.
20. Qvist T, Pressler T, Taylor-Robinson D, Katzenstein TL, Høiby N. Serodiagnosis of Mycobacterium abscessus complex infection in cystic fibrosis. Eur Respir J 2015;46:707–16.
21. Høiby N, Pressler T. Emerging pathogens in cystic fibrosis. . In: AK Webb, F Ratjen (eds). European Respiratory Monograph in Cystic Fibrosis 2006;11:66–78.
22. Aasnæs K, Johansen HK, Poulsen SS, Pressler T, von Buchwald C, Høiby N. Secretory IgA as a diagnostic tool for early Pseudomonas aeruginosa colonization. J Cystic Fibrosis 2013;12:81–7.
23. Johansen HK, Aanaes K, Pressler T, Nielsen KG, Fisker J, Skov M, et al. Colonization and infection of the paranasal sinuses in cystic fibrosis patients is accompanied by a reduced PMN response. J Cystic Fibrosis 2012;11:525–31.
24. Gacesa P, Russell NJ. The structure and properties of alginate. In: Gacesa P, Russell NJ, editors. Pseudomonas Infections and Alginates Biochemistry, Genetics and Pathology. London: Chapman and Hall, 1990: 29–49.
25. Rybtke MT, Jensen PØ, Høiby N, Tolker-Nielsen T, Bjarnsholt T. The implication of Pseudomonas aeruginosa biofilms in infections. Inflamm Allergy Drug Targets 2011;10:141–57.
26. Ciofu O, Johanneson M, Hermansen NO, Meyer P, Høiby N. Investigation of the *algT* operon sequence in mucoid and non-mucoid P. aeruginosa isolates from 115 Scandinavian patients with cystic fibrosis and in 88 *in vitro* revertants. Microbioloy 2008;154:103–13.
27. Pedersen SS, Kharazmi A, Espersen F, Høiby N. Pseudomonas aeruginosa alginate in Cystic Fibrosis sputum and the inflammatory response. Infect Immun 1990;58:3363–8.
28. Hansen N, Rasmussen A, Fiandaca M, Kragh K, Bjarnsholt T, Høiby N, et al. Rapid identification of Stenotrophomonas maltophilia by peptid nucleic acid fluorescence in situ hybridization. New Microbes New Infections 2014;2:79–81.
29. Høiby N, Collins MT, Espersen F, Hertz JB, Hoff GE, Schiøtz PO. Taxonomic application of crossed immunoelectrophoresis. Int J Syst Bact 1987;37:229–40.
30. Hoffmann N, Lee B, Hentzer M, Rasmussen TB, Song Z, Johansen HK, et al. Azithromycin blocks quorum sensing and alginat polymer formation and increases the sensitivity to serum and stationary gowth phase killing of P. aeruginosa and attenuates chronic P. aeruginosa lung infection in $Cftr^{-/-}$ mice. Antimicrob Agents Chemother 2007;51:3677–87.
31. Yang L, Haagensen JAJ, Jelsbak L, Johansen HK, Sternberg C, Høiby N, et al. In situ growth rates and biofilm development of Pseudomonas aeruginosa populations in chronic lung infection. J Bacteriol 2008;190:2767–76.