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Qualitative and quantitative agar invasion test based on bacterial colony/biofilm

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ABSTRACT

Invasion of the culture medium is a feature frequently studied in yeasts, in which it has been related to a greater virulence, but it is practically unknown in bacteria. Recently, it has been demonstrated that several clinically relevant bacterial species were also able of invading agar media, so it was necessary to design a microbiological assay to study the expression of this character in bacteria. Accordingly, a bacterial agar invasion test based on colony/biofilm development was designed, which allows qualitative and quantitative characterization of bacterial growth into the agar culture medium. Once the culture conditions were optimized, the test was applied to 90 strains from nine bacterial species, validating its usefulness for differentiating invasive strains (positive) from those non invasive (negative). The test also allows sorting invasive strains according to agar invasion intensity (low, moderate, high) and topographic invasion pattern (peripheral, homogeneous, mixed). Moreover, an image analysis routine to quantify the invasion was developed. Implemented method enables direct measuring of two invasion parameters (invasion area and number of invasion dots), automated calculation of three relative variables (invasion relative area, invasion dots relative density, and invasion dot average area), and the establishment of strain specific frequency histograms.
This new methodology is simple, fast, reproducible, objective, inexpensive and can be used to study a great number of specimens simultaneously, all of which make it suitable for incorporation to the routine of any microbiology laboratory. It could also be a useful tool for additional studies related to clinical aspects of bacterial isolates such as virulence and antimicrobial response.

1. Introduction

Microbiology is changing its approach to the bacterial study from the free-floating bacteria, or so called planktonic bacteria, to the organized bacteria grouped into communities called biofilms (Hall-Stoodly et al., 2004; Shapiro, 1998), since they are directly responsible for a great variety of infectious processes (Donlan, 2008).

Currently research is being geared on genotypic and phenotypic plasticity of the different functional subpopulations present in a biofilm, in order to identify virulence factors that could bring additional information about the possible behavior of bacterial isolates with clinical interest.

Yeast cells display various differentiation modes according to nutrient availability and environmental conditions, and there is some correlation between filamentation and invasive behavior (Casalone et al., 2005; Gagiano et al., 2002; Guldal and Broach, 2006). Since both *in vitro* culture medium invasion and *in vivo* tissues colonization by *Candida* spp. are performed by pseudomycelium formation, growth within the agar thickness has been considered as a putative virulence factor (Lim et al., 2012; Wächtler et al., 2012). By contrast, until recently there were very few bacterial species known to invade culture media, sometimes because they grew eroding the agar, as *Eikenella corrodens* (Chen and Wilson, 1992), and others because they were able to grow in the thickness of the agar, as actinomycetes species (Blix et al., 1990; Chater et al., 2010) and *Mycoplasma* species (Razin and Oliver, 1961). Given the rarity of this feature in bacteria, it has been only used as an additional macroscopic distinct property in description of the colony growth to distinguish phenotypically those species. However, in recent investigations on colonial architecture of bacterial species with clinical interest, our group has observed this feature with remarkable frequency in colonies of medium- and long-term evolution of species not recognized as invasive (Gómez-Aguado et al., 2013). Thus, by means of histological techniques, solid culture media invasion has been described as a phenotypic character discriminating between strains of a wide range of both gram-positive and gram-negative species, and it has been

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hypothesized that the expression of this phenotype could be associated with increased pathogenicity, as in yeasts (Gómez-Aguado et al., 2013).

To verify this hypothesis requires the study of a large number of strains recovered from different clinical infections and anatomical localizations. Nevertheless, the histological approach used to describe the phenomenon (Gómez-Aguado et al., 2011) does not seem the most appropriate methodology to study simultaneously a large number of specimens. In a recent review on static biofilms, Merritt et al. (2011) described a model to study the effects of antibiotic treatment in which colonies/biofilms are grown on semipermeable membranes that sit on agar plates. Modification of this model by removing the semipermeable membrane and enabling direct growth of colony/biofilm on the agar could provide the basis to develop a useful methodology to study the invasion. Accordingly, the aim of this work was to standardize an agar invasion test based on colony/biofilm growth allowing to characterize and quantify culture medium invasion on a routine manner, providing high repeatability and fast performance.

2. Materials and methods

2.1. Strains

To standardize the optimal culture conditions for carrying out a routine agar invasion test, two strains of *Staphylococcus aureus*, two strains of *Enterococcus faecalis* and two strains of *Escherichia coli* were used. These isolates had been used previously in another study (Gómez-Aguado et al., 2013). One isolate from each species was known as agar invasive, and the other as non-invasive.

The standardized agar invasion test was then applied to 90 isolates from the strain collection at the Microbiology Department, Complutense University of Madrid, corresponding to ten isolates from each of the following bacterial species: *S. aureus*, *Staphylococcus epidermidis*, *E. faecalis*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *E. coli*, *Salmonella typhi* and *Klebsiella pneumoniae*.

For the purpose of the tests, bacterial suspensions of each strain were prepared in sterile water from overnight cultures on Columbia Blood Agar (Biomerieux, France).

2.2. Agar invasion test

2.2.1. Agar invasion test standardization

We performed and compared several culture settings for each of the six strains included in the study. Three, five and ten microliters of 10^5 and 10^7 CFU/mL bacterial suspensions were inoculated on nutrient agar, Mueller-Hinton agar and Columbia Blood Agar. Each inoculum was spotted onto the surface of solid culture medium in triplicate (three spots in a row), and three different inocula were grown in each plate forming a 3 × 3 square (9 spots/plate). The plates were incubated for 3, 4, 5 and 6 days at 35 °C under aerobic conditions and constant humidity.

After the corresponding incubation period, bacterial biofilm was removed from the agar surface, without disrupting it, by rubbing the plate with a gloved finger while rinsing under deionized water. Then, plates were flooded with 0.5% methylene blue during 2 min to permit dye penetration within the culture medium and excess were removed by decantation. Finally, plates were visualized with a stereo microscope (Leica M80) to assess bacterial growth within the agar thickness.

2.2.2. Agar invasion test application

After having settled the optimal culture conditions, it was proceeded to inoculate the 90 strains included in the study. Specifically, 5 μL of 10^5 CFU/mL bacterial suspensions was dropped onto Mueller-Hinton agar following the 3 × 3 square model (3 spots per strain and three strains per plate). After 4 days at 35 °C, plates were washed and stained as described in Section 2.2.1, and then agar invasion was analyzed with a stereo microscope by two independent observers.

2.3. Histological control of invasion

To validate the agar invasion test it was necessary to verify that structures observed within the agar thickness, once the plates had been washed and stained, corresponded to bacterial growth and were not dye artifacts or culture medium crystals. For this purpose, two additional plates with spots of the three invasive strains used for standardization of the test were prepared and cultured according to the optimal culture settings (see Section 2.2.2). After incubation, one plate was washed and stained with methylene blue, and the other plate was left untreated. Both plates were then processed following a previously described histological method (Gómez-Aguado et al., 2011). In brief, plates were flooded with 2% agar at 45 °C and left to cool down until solidified. Then, agar blocks were taken from the bacterial growth areas, fixed by immersion in 2.5% glutaraldehyde for 2 h, postfixed in 1% osmium tetroxide and embedded in epoxy resin (Eponate 12, Ted Pella Inc., CA, USA) according to the manufacturer recommendations. Semithin sections (0.5 μm thick) were stained with 1% toluidine blue and observed with a light microscope (Leica, DM5000). Ultrathin sections (50 nm thick) were stained with Reynold's lead citrate and observed with an electron microscope (Hitachi H-7000).

2.4. Image analysis

To check if an objective quantification of the invasion was possible, a measurement routine was designed by using an image analysis software (Leica QWin).

Four *E. faecalis* strains (EF26, EF40, EF45, and EF95) positive to agar invasion test and showing different invasion patterns were selected. Each isolate was plated and cultured according to the optimal culture settings registered in Section 2.2.2, following the 3 × 3 square model, and culturing three plates for each strain (a total of 9 spots per strain). Plates were photographed at the end of the incubation period with a conventional digital camera, including a gage-rule for size control. These images were used for quantification of bacterial growth on the agar surface. Each colony/biofilm presented in the plate was individualized by means of real color detection and binary segmentation. Then, measurements of maximum diameter in mm (D_M) and total area in mm^2 (A_T) were performed.

Plates were then washed and stained with 0.5% methylene blue. Agar invasion was visualized with 5× objective of a light microscope with motorized stage (Leica DM5000B) and captured with a digital camera (Leica DC300) coupled to microscope after subtraction of unspecific blue background produced by the dye. Bacterial growth into the culture medium thickness was extracted by means of real color detection and segmentation. Then, total invasion area in mm^2 (A_I) was measured and invasion dots were counted (ID_T). Moreover, individualized measurement of the area occupied by each invasion dot in μm^2 was carried out, and a frequency histogram (invasion histogram) was developed which clustered invasion dots into three classes according to size: less than 500 μm^2, between 500 and 1500 μm^2, and greater than 1500 μm^2.

Finally, three parameters were calculated for each spot: a) relative invasion area in percentage (A_R) respect to the total area occupied by the colony/biofilm on the agar surface, as $A_R = A_I \times 100 / A_T$; b) invasion dots relative density (invasions/mm^2), as $ID_R = ID_T / A_T$; c) invasion dot average area in μm^2, as $A_{ID} = A_I \times 10^6 / ID_T$.

Nine spots were analyzed for each strain. Thus, the means of the nine measures were considered as final strain values. The test precision and reproducibility were established by calculating standard deviations and coefficients of variation.