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Comparison of the InoqulA and the WASP automated systems with manual inoculation

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Abstract

The quality of sample inoculation is critical to achieve optimal yield of discrete colonies in both monomicrobial and polymicrobial samples to perform identification and antibiotic susceptibility testing. Consequently, we compared the performance between the InoqulA (BD Kiestra), the WASP (Copan) and manual inoculation. Defined mono- and polymicrobial samples of 4 bacterial species as well as cloudy urines were inoculated on chromogenic agar by the Inoqula, the WASP and manually. Images taken with ImagA (BD Kiestra) were analyzed with the Vision Lab v3.43 image analysis software to assess the quality growth and to prevent subjective interpretation.

A 3 to 10 fold higher yield of discrete colonies was observed following automated inoculation with both the Inoqula and the WASP systems compared to manual inoculation. The difference of performance between automated and manual inoculation was mainly observed at concentrations higher than 10^6 bacteria/ml. The inoculation with the InoqulA allowed to obtain significantly more discrete colonies than the WASP at concentration above 10^7 bacteria/ml. However, the level of difference observed was bacterial species-dependent. Discrete colonies of bacteria present in 100 to 1000 fold lower concentrations than the most concentrated populations in defined polymicrobial samples were not reproducibly recovered, even with automated systems. The analysis of cloudy urines showed that the InoqulA inoculation provided a statistically higher number of discrete colonies compared to WASP and manual inoculation. Consequently, the automated InoqulA inoculation greatly decreased the requirement of bacterial subculture and thus resulted in a significant reduction of time-to-results, laboratory workload and laboratory costs.

Introduction

The emergence of automation in bacteriology opens a new era in clinical diagnostic laboratories. Automation is impacting the management and the laboratory workflow but also offers new perspectives for research and development in bacteriology by developing intelligent algorithms and driving innovation. Sample inoculation is a fastidious and repetitive process representing about 25% of a laboratory's workload (1). Thus, automated inoculation systems represent a need in diagnostic laboratories given the reduction of human, material and financial resources and the increase in sample volumes (1). Moreover, the quality of inoculation is critical to achieve optimal yield of discrete colonies in both monomicrobial and polymicrobial samples to facilitate rapid identification (ID) and antibiotic susceptibility testing (AST). Several inoculation and streaking instruments are currently available for routine diagnostic laboratories including the Autoplak (NTE-SENER), the InoqulA (BD Kiestra), the Innova (BD), the PreLUD (I2A), the Previ-Isola (bioMérieux) and the WASP (Copan). However, the true effectiveness of automated inoculation systems needs to be validated by independent routine clinical microbiology laboratories. Compared to manual streaking, a few studies have demonstrated that the InoqulA and PreviIsola automated systems produced more isolated colonies, showed better reproducibility, no cross-contamination and exhibited a significant decrease in hands on plating time (2-5). These studies concluded that such automated systems should improve the laboratory workflow and shorten the time-to results but direct laboratory impact assessments remain to be performed to confirm these expectations. Moreover, the available few studies only compared automated to manual streaking performance but direct comparative studies between available automated systems remained to be performed.

Consequently, we compared the performance between manual inoculation, the automated inoculation systems InoqulA BT systems (BD Kiestra, Netherlands) and the Walk Away Specimen Processor (WASP, Copan, Italy). Several parameters including the yield of discrete colonies and colony distribution were determined following inoculation of monomicrobial and polymicrobial defined samples. Moreover, the capacity of each inoculation system to reproducibly produce discrete colonies and the requirement to perform additional re-isolation to obtain discrete colonies for subsequent ID and AST were prospectively evaluated on clinical cloudy urines samples. The need for re-isolation, time-to-results and laboratory analytical costs

were determined to assess whether the performance of the different inoculation systems has an impact on laboratory financial and time-to-results outcomes.

Materials AND methods

Strains, media and bacterial suspension

Escherichia coli strain ATCC 25922, *Klebsiella pneumoniae* strain ATCC BAA-1706, *Staphylococcus aureus* strain ATCC 29213 and *Enterococcus faecalis* strain ATCC 29212 were grown on Columbia agar with 5% sheep blood (BD columbia III agar, BD, Franklin Lakes, NJ, USA) at 37°C in 5% CO_2 atmosphere incubators. Colonies of each bacterial species were utilized to prepare a bacterial suspension in saline solution adjusted to a 0.5 McFarland turbidity measured with a Densitometer Densicheck instrument (Biomérieux, Marcy-l'Etoile, France) and corresponding to a bacterial concentration of 10^8 colony forming units per ml (CFU/ml). Different concentrations of monomicrobial suspension in saline solution ranging from 10^8 to 10^3 CFU/ml were prepared by doing serial 10-fold dilutions in saline solution. All bacterial suspensions were plated on Columbia agar with 5% sheep blood to verify the number of CFU/ml. Polymicrobial suspensions containing 4 bacterial species at different ratio ranging from 1:1 to 1:1000 (supplementary table 1) were obtained by mixing different concentrations of the diluted and non-diluted monomicrobial suspensions.

Sample collection

Cloudy urine samples were collected during a one month period from ambulatory and hospitalized patients at the University Hospital of Lausanne (Switzerland). A total of 75 cloudy urines found positive for bacteria by Gram staining microscopy were selected to only include in the study urinary samples containing at least 10^5 to 10^6 CFU/ml. Selected urinary samples were transferred into sterile 5 ml Copan tubes (Copan, Brescia, Italy), vortexed and inoculated with the WASP, the InoqulA BT or manually as described below.

Inoculation and incubation

According to specific guidelines for urine cultures (6-9), detection at the level of 10^2 CFU/ml is necessary for specific populations such as women with acute cystitis, catheterized specimens and patients in the early development of a urinary tract infection. Thus, the guidelines specifically state that at least 10 µl of urine should be plated using a back and forth streaking method to detect most of clinically relevant urinary tract infections. Therefore, inocula of 10 µl were streaked onto chromogenic agar (BBL CHROMagar Orientation, BD, Franklin Lakes, NJ, USA) manually and with the automated inoculation systems InoqulA BT and WASP, respectively.

Chromogenic agar is routinely used in many diagnostic laboratories for the analysis of urine samples and facilitate the recognition and classification of bacterial colonies by the BD Vision Toolbox with embedded Vision Lab v3.43 imaging analysis software.

Manual and WASP streaking were performed with a 10 µl loop whereas plate inoculation with the InoqulA was performed with a rolling magnetic bead. The volume of 10 µl was seeded onto chromogenic agar with a calibrated pipette for manual streaking and the InoqulA automated system and with a 10 µl loop for the WASP automated system. Two manual quantitative plate inoculation patterns were performed by an experienced microbiologist with 10 µl loops following (1) zig-zag streaking pattern (MAN1) and (2) a central single streaking throughout the plate followed by a zig-zag pattern (MAN2) (figure1). Two similar automated quantitative plate inoculation patterns with the InoqulA BT and the WASP were performed following (1) a zig-zag streaking pattern (INO1, WAS1) and (2) a central single streaking of 20 mm followed by a zig-zag pattern (INO2 and WAS2) (figure 1).

The manual MAN2 streaking pattern is a conventional semi-quantitative approach used routinely by many diagnostic laboratories. The INO1 and WAS2 streaking approaches are semi-quantitative patterns recommended by the manufacturers (BD and Copan, respectively) to obtain optimal quantitative and qualitative results. The INO2 is similar to the WAS2 streaking pattern whereas the WAS1 and MAN1 are similar to the INO1 streaking pattern. Thus, the INO2, WAS1 and MAN1 streaking patterns were chosen to use similar streaking approaches required for direct comparison of the inoculation efficiency of the manual and automated systems.

The inoculated chromogenic agar plates were incubated in a normal ambient atmosphere for 20 hours at 35°C allowing to obtain both an acceptable turn-around-time (TAT) and enough microbiological material to perform ID and AST. Automated and manual inoculations of defined monomicrobial and polymicrobial samples were performed at least in three independent experimental runs whereas inoculation of cloudy urines was performed once per sample.

Analysis of reporting times and laboratory costs

The time to report results and laboratory costs were calculated based on the ability of the different systems to produce a minimal number of isolated colonies to perform identification by MALDI-TOF and AST. The minimal number of *E. coli* colonies grown on chromogenic BBL CHROMagar Orientation agar plates in a normal ambient atmosphere for 20 hours at 35°C was determined following conventional laboratory procedures with a minimum of 1 discrete colony required for ID by MALDI-TOF and a minimum of 5 colonies required to make a 2ml bacterial suspension in saline solution with a turbidity of 0.5 McFarland for AST. A delayed time to report result of 1 working day (16hr to 24 hr) and additional laboratory costs were applied when the minimal number of isolated colonies required to perform the ID and AST procedures was not obtained. The laboratory cost per re-isolation was calculated in both Swiss Francs (CHF) and European Euros (EUR) based on consumable prices and labor costs including social security charges applied at the university hospital of Lausanne, Switzerland, as follows: Agar plate (1 CHF / 0.8 EUR), plastic loop (0.1 CHF / 0.1 EUR), 5 min working time to perform a re-isolation (5.9 CHF / 4.9 EUR) for a total of 7 CHF or 5.8 EUR per re-isolation. The conversion rate of 1 EUR = 1.2 CHF was calculated in November 2014 and may be subjected to variations due to the volatility of the foreign exchange rate. The experimental working time of 5 min to perform re-isolation includes the following tasks: (1) Collect the agar plate containing the sample to re-isolate in the incubator, (2) collect a sterile plate for subculture in the cold room (3) collect a plastic loop, (4) plate labelling, (5) colony picking and 4 quadrant plate streaking, (6) store the plates into the incubators. The working time of 5 min was measured and used for an experimental modelization of additional laboratory costs due to the requirement of subculture to perform ID and AST from discrete colonies in both automated and conventional laboratories. The measured working time strongly depends on the organisation of the laboratory workflow and may greatly vary between laboratories.

Imaging and Image analysis

All images were taken using a specialized imaging device called the ImagA BT (BD Kiestra) which allowed us to obtain reproducible and consistent images with the different inoculation methods and sample preparations. The resolution of the camera allowed recognition of objects equal or bigger than 0.4 mm diameter. Objects below 0.4 mm were thus considered as small

noisy objects. Image analysis was performed with the Vision Lab v3.43 software (Van Loosdrecht Machine Vision BV, Buitenpost, The Netherlands). Image analysis was used to provide a reliable and objective measure for the properties of the colonies, minimizing the bias from manual observation. Parameters of the image analysis software were trained by an experienced lab technician by selecting objects and specifying their discreteness and bacterial species. Properties of colonies were automatically measured with the Vision Lab v3.43 software enabling fast automated counting of discrete colonies and automatic recognition of specific bacterial species.

The Hough circle transform (10) was used as a robust method giving the correct position of the Petri-dish for every image in the set (figure 2A and 2B). Because of refractions and reflections at the dish border, a few millimetres of the outer border of the Petri-dish image were ignored by the image analysis software to increase measurement accuracy. The size of this border is equal among all images preventing any bias towards any image (figure 2B). Pixels with a high saturation value of 70 were considered as non-white and were selected as object pixels (figure 2C). A lower value resulted in more growth pixels around each colony while a higher value resulted in less growth. As a result, a white agar background was required for a reliable detection of bacterial growth.

Each image contained a white agar background with colonies of bacterial species exhibiting different colours. Image features from each object in the Petri-dish image were calculated. Geometric features were used to determine colonies discreteness (discrete, non-discrete) and colour features were used to determine bacterial colony species. These geometric and colour features were used to automatically classify discreteness and bacterial colony species, respectively. In addition, every object smaller than 0.4 mm was not considered as a bacterial colony and was removed.

Classification of discreteness and bacterial species was done by a Linear Discriminant Analysis (LDA) (11, 12) based classifier. LDA is a linear model which uses statistics of the data to determine the optimal separation between the different classes. A data set of 3379 images of discrete and non-discrete colonies of *E. coli, K. pneumoniae, S. aureus* and *E. faecalis* were defined by a technician resulting in the defined data set. The LDA classifier was trained with samples from the defined data set meaning that colony discreteness and species recognition was

191 determined indirectly by the lab technician and not by the specific configuration of the image
192 analysis software.

193 The LDA classifier for determining colony discreteness was trained and evaluated to classify
194 objects into discrete and non-discrete colonies based on their geometric features (figure 2D). A
195 linear transformation of geometric features was automatically determined by LDA by using the
196 defined data set. LDA minimizes the variance within a class and maximizes variance between
197 classes allowing the formation of clusters. Highly separated clusters yields high classification
198 accuracy. Each object's features were transformed to the trained LDA space to form the discrete
199 and non-discrete clusters. The closest cluster was chosen as the proper class for each object. The
200 real class for each object in the evaluation set was known (e.g. discrete or non-discrete) so results
201 of the classification could be compared to the defined data set.

202 The LDA classifier for determining colony species was trained and evaluated to classify discrete
203 colonies of *E. coli*, *K. pneumoniae*, *E. faecalis* and *S. aureus* based on the colour features of the
204 discrete colony. A linear transformation of colour features was automatically determined by the
205 LDA using the defined data set favouring high cluster separation. Colour features of each
206 discrete colony in the defined data set were transformed to the trained LDA space resulting in
207 four clusters, one for each bacterial species (figure 2E). The closest cluster was chosen as the
208 proper class for each discrete colony. The real class for each discrete colony was known and the
209 results of the classification could be compared to the defined data set.

210 The accuracy of LDA classifiers were defined as the percent of colonies correctly classified
211 compared to the defined data set compiled by a technician. A quantitative analysis of the
212 evaluation results provided insight into the error that could be expected from the measurements
213 (see Results). The error of classification was similar for each inoculation method and did not bias
214 the results for any specific automated or manual inoculation method.

215 Median discrete colonies distribution was determined as follows. The media plate was delimited
216 in 1500 lines starting from the border located close to the sample seeding zone (line 0) to the
217 opposite plate border (line 1500). For each line the number of discrete colony pixels on that line
218 was divided by the total number of growth pixels on that line giving a normalized measure of the

219 percentage of discrete colonies on each line. Finally, the concatenation of all lines was plotted
220 for each inoculation method and for each bacterial species.

221 **Statistical analysis**

222 The statistical difference of the number of discrete colonies obtained following automated and
223 manual inoculation of monomicrobial and polymicrobial samples were analyzed by multiple
224 comparisons of means using contrasts in linear regression in R. The analysis was done using the
225 lm() function in R followed by the extraction of contrasts using the contrast() function from
226 Package contrast and the multiple comparisons including the confidence intervals around the
227 differences between means were computed by the glht() function from Package multcomp.

228

229 A one way ANOVA multiple comparisons test was performed using the GraphPad Prism 6.04
230 software to analyze the statistical difference of the number of discrete colonies obtained from
231 cloudy urine samples with the automated and manual inoculations.

Results

Image analysis

The image analysis was performed in 5 steps as shown in figure 2. First, the Petri-dish surface was determined by using a Hough circle transform (10) which was specifically suited for detecting circles (figure 2B). Secondly, bacterial growth was determined by selecting pixels with high colour saturation (e.g. if the colour is different from white) (figure 2C) (13). Connected pixels were grouped into objects and objects smaller than 0.4 mm were considered as noise and were removed. Consequently, a group of connected pixels with high colour saturation were considered as growth and called an object. Then, each object could either be one discrete colony or several connected colonies. Discrete objects were recognized by the discreteness LDA classifier. Objects with a diameter below 0.4 mm and/or with a distance to the nearest growth below 1 mm were removed. All remaining objects were considered as discrete colonies (figure 2D). Finally, the bacterial species of each discrete colony was determined by a bacterial species LDA classifier trained with four bacterial species, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Staphylococcus aureus* (figure 2E). Consequently, in this study, only these four bacterial species could be automatically recognized on the agar plates.

An evaluation of the accuracy to correctly classify discrete and non-discrete objects as well as bacterial colony species was performed for each step involved in the image analysis process (figure 2C to 2E). The evaluation was performed using a defined data set containing 3379 images of known objects belonging to discreteness (discrete, non-discrete) and bacterial species classes (1915 Non-discrete objects, 423 *Escherichi coli*, 353 *Klebsiella pneumoniae*, 199 *Enteroccocus faecalis* and 489 *Staphylococcus aureus*). Objects from all the bacterial species classes were discrete. The defined data set was divided in a training set and an evaluation set to be used for a two-fold cross validation.

The discreteness LDA classifier was trained and evaluated to classify objects into discrete and non-discrete colonies based on their geometric features (figure 2D). Compared to the defined data set characterized by a trained technician, 92% of the objects were correctly classified as discrete or non-discrete objects by the LDA classifier giving thus a 92% probability of correct automated discreteness classification of undefined samples. Then, the bacterial species LDA classifier was trained and evaluated. Discrete colonies were classified as *E. coli*, *K. pneumoniae*,

E. faecalis and *S. aureus* based on the colour features of the discrete colony. Compared to the defined data set characterized by a trained technician, 99.9% of the bacterial species colonies were correctly classified by the LDA classifier giving thus a 99.9% probability of correct automated bacterial species colony classification of undefined samples.

Thus, the accuracy of both the discreteness classifier and the bacterial species classifier resulted in reliable measurement results for the properties of the colonies.

Quality of isolation of different bacterial concentrations of *E. coli*.

Quantitative streaking patterns used in this study are routinely performed with urinary samples which require quantification of growing microorganisms for biomedical interpretation. *E. coli* is the most prevalent etiological agent of urinary tract infection (UTI) ranging from 66% to 90% of cases in complicated and uncomplicated UTI, respectively (9). The quality of isolation was thus assessed with different bacterial concentration of *E. coli* ranging from 10^3 to 10^8 CFU/ml to measure the ability of the different systems to generate discrete colonies on a wide range of bacterial concentrations. Bacterial colonies bigger than 0.4 mm and distant to 1 mm or more from the nearest growth were considered as discrete colonies (figure 2D). These criteria were chosen to ensure that manual or automated colony picking can be easily performed without risk of contamination with nearby bacterial growth.

The different inoculation methods showed a gradual increase in the number of discrete colonies with rising bacterial concentrations but differed by reaching a peak or a plateau of isolated colonies at different bacterial titers (figure 3 and supplementary figure 1). A gradual increase of discrete colonies reaching a plateau at 10^7 CFU/ml was observed with the INO1 inoculation. The INO2 inoculation was able to generate more isolated colonies than the INO1 at lower bacterial concentrations producing thus a high yield of discrete colonies on a wider range of bacterial concentrations. The MAN1 streaking showed a weak gradual increase of isolated colonies with rising bacterial concentrations to reach a maximal median value at 10^8 CFU/ml. A high yield of discrete colonies was obtained with the MAN2 streaking at low to moderate bacterial concentrations but a significant decreased performance was observed at high bacterial concentrations. Similarly, the WAS1 and WAS2 inoculation showed an increased yield of discrete colonies but exhibited a weak performance at 10^8 CFU/ml. Thus, the INO1, INO2 and MAN1 inoculations showed a gradual increased of isolated colonies reaching a plateau of

discrete colonies at different bacterial concentrations whereas the MAN2, WAS1 and WAS2 inoculation methods were characterized by an increased yield of discrete colonies followed by a significant reduced performance when reaching moderate (10^6/ml with the MAN2) to high bacterial concentrations (10^7/ml with the WAS1 and WAS2 inoculations), respectively. The automated inoculation systems InoqulA and WASP showed a statistically significant higher yield of discrete colonies ($p < 0.05$, multiple comparisons of means) than manual inoculation at 10^7 CFU/ml whereas the InoqulA produced statistically more discrete colonies ($p < 0.05$, multiple comparisons of means) than the WASP and manual inoculation at 10^8 CFU/ml (supplementary table 2).

Quality of isolation of defined monomicrobial samples

As demonstrated with the inoculation of different bacterial concentrations of *E. coli* (figure 3), a significant difference between the inoculation systems was mainly observed at bacterial concentrations $\geq 10^7$ CFU/ml. The streaking quality of manual and automated inoculation was thus assessed by measuring the yield of discrete colonies following inoculation of four bacterial species suspensions at a concentration of 10^8 CFU/ml. Two Gram negative and two Gram positive bacteria, *Escherichia coli, Klebsiella pneumoniae, Staphyloccocus aureus* and *Enterococcus faecalis*, were used to integrate morphological and physiological traits differences that may impact the streaking efficiency of the manual and automated systems. In addition, colonies of these 4 bacterial species growing on chromogenic agar exhibit different colours that facilitate the recognition and classification of discrete colonies by the Vision Lab v3.43 software. The yield of discrete colonies and the differences observed between the automated and manual inoculations were bacterial species dependent (figure 4). All the streaking methods except MAN2 were able to produce a high yield of discrete colonies of *E. faecalis*. However, the INO1 inoculation produced a statistically higher number of discrete colonies ($p < 0.05$, multiple comparisons of means) than manual and WAS1 inoculations (supplementary table 3). To the contrary, a lower yield of *K. pneumoniae* isolated colonies was obtained with the 6 streaking approaches compared to the other bacterial species, with no statistical difference between automated and manual inoculations (supplementary table 3). The yield of discrete colonies of *E. coli* and to a lesser extend of *S. aureus* was strongly dependent on the streaking method. A statistically significant higher yield of *E. coli* discrete colonies ($p < 0.05$, multiple comparisons

of means) was reproducibly obtained with the InoqulA instrument compared to manual or WASP plate streaking (figure 4 and supplementary table 3). A high yield of *S. aureus* discrete colonies was obtained with the InoqulA and with the WAS2 streaking methods whereas a poor to low number of isolated colonies was obtained manually or with the WAS1 streaking approaches. However, only the INO1 inoculation exhibited a statistically significant higher yield of *S. aureus* discrete colonies ($p < 0.05$, multiple comparisons of means) compared to manual and WAS1 inoculations (supplementary table 3).

Automated and manual streaking approaches exhibited different discrete colony distribution patterns (supplementary figure 2A and B). A gradual increase in the number of discrete colonies following the inoculation path throughout the plate was observed with the InoqulA and with the WAS2 streaking approaches. Interestingly, the InoqulA magnetic bead inoculation method showed a larger zone of discrete colony distributions due to its capacity to cover the entire surface of the plate compared to manual or WASP loop streaking that have a limited access to the plate edges (figure 1 and supplementary figures 1 and 2). Identical patterns of distribution were observed between the different tested bacterial species except for *K. pneumonia*e. Unlike manual streaking, the distribution of *K. pneumoniae* with the WASP and InoqulA automated inoculations differed by showing a later appearances of discrete colonies following the path of the streaking pattern when compared to other tested bacterial species (supplementary figure 2B and data not shown).

Quality of isolation of defined polymicrobial samples

The ability of the different inoculation systems to obtain discrete colonies of each bacterial species contained in a polymicrobial sample was assessed to determinate their discriminative power. Eleven polymicrobial suspensions containing *E.coli*, *K. pneumoniae*, *S. aureus* and *E. faecalis* were obtained by mixing the 4 bacterial species at different ratio ranging from 1:1 to 1000:1 between the highest and lowest bacterial concentrations (supplementary table 1). The results obtained with mixes M01 to M10 (supplementary figure 3) were similar to those observed in the polymicrobial suspensions mix M11 (figure 5). The mix M11 was composed of *E. faecalis* at 10^7 CFU/ml, *S. aureus* at 10^6 CFU/ml, *E. coli* at 10^5 CFU/ml and *K. pneumoniae* at 10^4 CFU/ml. The InoqulA and the WASP inoculation produced a statistically significant higher yield of *E. faecalis* discrete colonies ($p < 0.05$, multiple comparisons of means) compared to manual

streaking (supplementary table 4). However, the 6 inoculation methods produced a low yield of colonies of *S. aureus* which was present at a 10 fold lower concentrations than *E. faecalis*. In addition, no significant statistical differences was observed between the automated and manual inoculation approaches (supplementary table 4). Discrete colonies of *E. coli* and *K. pneumoniae* present at 100 to 1000 fold lower concentrations than the most concentrated *E. faecalis* populations in the sample were not reproducibly recovered neither with the manual nor with the automated inoculations methods used in this study. Thus, the results of the MI01 to MI11 suggest that colonies of bacterial species present at 100 fold or lower concentrations than the most concentrated bacterial population in a polymicrobial sample are likely not recovered following manual or automated inoculation with the streaking patterns used in this study.

Performance of the manual and automated systems on clinical cloudy urines.

The performance of the different systems and their impact on the time to report results and on laboratory costs were assessed by determining (1) the yield of discrete colonies and (2) the need for re-isolation of colonies for identification (ID) by MALDI-TOF and antibiotic susceptibility testing (AST). A total of 75 cloudy urines defined as positive by Gram stain results were prospectively inoculated manually or with the InoqulA and the WASP automated systems. Among them, 41 urines only positive for *E. coli* were analyzed with the Vision Lab v3.43 software to determine the yield of discrete colonies obtained by each inoculation system (figure 6). The remaining 34 urines considered as contaminated urines or including bacterial species not recognized by the Vision Lab v3.43 software were excluded from the analysis. The INO1 inoculation showed a statistically significant higher yield of discrete colonies ($p < 0.05$, one way ANOVA multiple comparison) compared to the manual and WASP plate streaking (figure 6 and table 1) whereas no significant difference was observed between manual, INO2 and WASP inoculations. One discrete colony of *E. coli* grown on BBL chromogenic agar was required to perform identification by MALDI-TOF whereas 5 discrete colonies of *E. coli* were required to prepare a bacterial suspension in 2ml saline solution with a turbidity of 0.5 McFarland to perform an AST as recommended in the EUCAST/CLSI guidelines. The additional laboratory cost per re-isolation including consumables and technician time was estimated to be equal to 5.8 EUR. According to these parameters, the number of discrete colonies grown on BBL chromogenic agar following manual and automated inoculation was measured to assess the need for re-isolation resulting in delayed time to report results of 1 working day (16h to 24h) and

additional laboratory costs (figure 7A and B). All the inoculation methods except the INO1 required re-isolation for bacterial identification for 3 (7.3%) to 8 (19.5%) cloudy urines samples. Moreover, the additional laboratory costs due to re-isolations for bacterial ID ranged between 17.5 to 46.7 EUR, which represent an additional cost of 43 to 114 EUR when extrapolated to 100 samples for simplicity (figure 7A). The InoqulA INO1 inoculation showed also the best performance by requiring re-isolation of only 4 out of 41 (9.8%) cloudy urines samples to perform ID and AST (figure 7B). Re-isolation with the other inoculations methods was required for 10 (24.4 %) cloudy urines with the INO2 to 24 (58.5%) cloudy urines with the MAN2. A similar level of performance was observed between the MAN1, WAS1 and WAS2 inoculations methods which showed a need of re-isolation for 15 (36.6%) to 18 (43.9%) cloudy urines samples. The laboratory costs due to re-isolation to perform ID and AST extrapolated to 100 samples showed a minimum laboratory cost of 57 EUR with the INO1 inoculation and a maximum laboratory cost of 342 EUR with the MAN2 streaking. Thus, a 2.5 (INO2) to 6 (MAN2) fold increase in laboratory costs was observed with the INO2, MAN1, MAN2, WAS1 and WAS2 inoculation methods compared to the INO1 inoculation method, which presented the best performance following semi-quantitative inoculation of clinical urinary samples.

Discussion

This is to our knowledge the first study comparing the performance of two automated systems, the WASP and the InoqulA, with manual inoculation on both defined and clinical samples. The quality of inoculation was assessed by measuring several parameters including the yield of isolated colonies and their distribution on the agar plates. Quality of inoculation is a critical factor in clinical bacteriology since a poor yield of discrete colonies significantly increases the time-to results, the hands-on-time and the costs by adding additional steps of manual colony isolation and subculture, which often prolong the time to identification and to antibiotic susceptibility testing results by 1 working day (16 to 24 hrs).

Images of the plates were taken with the ImagA BT digital imaging solution module (BD Kiestra) and were analyzed with Vision Lab v3.43 software to assess the quality of colony growth. Thus, the yield of discrete colonies and colony distribution were accurately measured by an image analysis software that removed the subjective interpretation of manual observation and

allowed a precise quantification of the streaking quality of the different automated and manual approaches used in this study.

Only semi-quantitative inoculation approaches were used in this study to determine the qualitative performance of the manual and automated quantitative streaking methods. The InoqulA INO1 pattern (zig-zag) and the WASP WAS2 pattern (20 mm central streaking followed by a zig-zag streaking) were used as optimized factory designed semi-quantitative inoculation protocols. The manual MAN2 streaking approach (central streaking throughout the plate followed by a zig-zag streaking) was chosen as the conventional semi-quantitative manual inoculation used in our diagnostic laboratories. The INO2, MAN1 and WAS1 were chosen to use similar inoculation protocols allowing direct comparison between the different automated and manual systems. Thus, all the results obtained in this study should not be extrapolated to other inoculation methods that may exhibit a higher performance in colony isolation such as the conventional non-quantitative 4 quadrants streaking methods including a sterilization of the loop after streaking of the first quadrant that can be easily performed manually and by the WASP system. Thus, laboratories should carefully select and validate automated qualitative and quantitative patterns yielding the best performance for each sample type.

Similar to previous studies (2-5, 14), a higher number of discrete colonies were reproducibly obtained with the automated inoculation system InoqulA and WASP compared to manual inoculation. Moreover, the difference of the recovery of microorganisms obtained between manual and automated inoculations increased with bacterial concentrations in the sample. The manual and automated inoculation approaches except MAN1 and WAS1 showed a similar performance with high recovery of discrete colonies at low to moderate bacterial concentration. However, the automated systems allowed a significantly higher recovery of discrete colonies compared to manual inoculation at high bacterial concentrations of about 10^7 CFU/ml. Moreover, only the InoqulA INO1 and INO2 were able to reproducibly generate high yield of discrete colonies at concentration above 10^7 CFU/ml with all bacterial species tested in this study. The WASP inoculation system exhibited a high performance up to 10^7 CFU/ml but was unable to allow efficient recovery of isolated colonies of some bacterial species at high bacterial concentrations. Using pure bacterial cultures, the difference of performance observed between the InoqulA, the WASP and manual inoculation was bacterial species-dependent. The InoqulA

INO1 and INO2 showed a significantly higher performance compared to manual and WASP streaking following inoculation of high concentration of *E. coli* and to a lesser extent of *K. pneumoniae* whereas no or little difference was observed between the InoqulA INO1/INO2 and the WASP WAS2 following the inoculation of a high concentration of *E. faecalis* or *S. aureus*. Thus, the efficiency of each inoculation method to generate isolated colonies relies on multiple factors including specific morphological and physiological traits of bacterial cells and colonies as well as the used inoculation technology. Bacterial cells membranes, shape and sizes likely exhibit different affinities for the inoculation support (magnetic beads, plastic or metal loops) and for the agar surface that may impact the release of microorganisms during the streaking or the rolling process and thus the distribution gradient and the yield of discrete colonies. For instance, we observed a slower release of the encapsulated *K. pneumoniae* strain by the InoqulA and the WASP system as compared to other species (supplementary figure 2B and data not shown), which resulted in a decreased yield of discrete colonies. This observation suggests that the capsular polysaccharide of *K. pneumoniae* may confer a stronger interaction of the bacteria with the inoculating device and thus decrease the rate of bacterial release during the streaking process. Moreover, bacterial colonies growth kinetics and sizes likely also impact the recovery of discrete colonies. Finally, the higher performance of the InoqulA INO1 with all bacterial species tested in this study is also likely based on its capacity to generate a gradual distribution of discrete colonies on a larger zone of the media plate compared to other streaking approaches as observed in figure 1 and in supplementary figures 1 and 2, optimizing thus the surface available for the recovery of isolated colonies.

None of the manual or automated inoculation system tested in this study allowed the recovery of discrete colonies of bacterial species present in 100 to 1000 fold lower concentrations than the most concentrated species present in the sample. These results suggest that only a minor fraction of bacterial species present in polymicrobial samples are identified by routine laboratory culture procedures. Missing "minority species" has in most cases a small impact on the clinical outcome since clinically-relevant infectious agents are most often present at similar or higher concentrations than other microorganisms present in polymicrobial samples such as urine sample. However, these results also indicate that the use of selective media in routine bacteriology is required to identify and recover true pathogens present in lower concentrations than the natural microflora in complex samples such as respiratory samples.

The quality of inoculation is characterized by the ability of a system to obtain a high yield of discrete colonies for each bacterial species of a monomicrobial or polymicrobial sample. However, the real impact of an inoculation system on laboratory results and thus on clinical outcomes is not based on its ability to generate a maximal amount of isolated colonies but mainly on its ability to produce a critical minimal amount of discrete colonies required to perform downstream applications including bacterial ID by MALDI-TOF, phenotypic and biochemical tests and AST. According to EUCAST (www.eucast.org) and CLSI guidelines but also in prediction of automatic colony picking technology, ID and AST should ideally be performed from isolated colonies and not from a bacterial lawn, even with pure culture. Thus, the impact of quality of inoculation on the time to report results and laboratory costs was assessed on clinical cloudy urines samples by determining the yield of discrete colonies and the need for re-isolation to perform ID and AST. These results showed that the InoqulA INO1 system produce a statistically higher yield of discrete colonies than manual and WASP inoculation but was also characterized by its higher ability to obtain the minimal amount of discrete colonies necessary to perform rapidly downstream applications. The INO1 was the only tested inoculation approach that allowed direct identification by MALDI-TOF of the 41 cloudy urines positive for *E. coli*. Moreover, subculture was required for only 4 out of 41 (9.7%) cloudy urines following INO1 inoculation indicating that AST could be performed directly for 37 (90.3 %) of the *E. coli* strains recovered in the urinary samples. Altogether, the conventional routine laboratory manual semi-quantitative approach exhibited the lowest performance, clearly indicating that automation may efficiently improve laboratory productivity while reducing laboratory cost. This study showed that the ability of the InoqulA INO1 to yield a high number of discrete colonies reduced the turn-around-time (TAT) compared to the other inoculation approaches allowing significant reduced laboratory costs by reducing the need to make bacterial subculture for ID and AST procedures. Moreover, the reduced TAT observed with the InoqulA automated system should positively impact clinical management and thus clinical costs. However, the hypothetical benefits remain to be addressed in a specific study measuring the impact of partial and full laboratory automation on clinical outcomes and hospitalization costs.

In summary, this study showed that a higher number of discrete colonies were reproducibly obtained with the InoqulA and WASP automated systems compared to manual inoculation. The InoqulA exhibited a higher performance compared to the WASP system at bacterial

concentration higher than 10^7 CFU/ml. However, the difference observed was bacterial species dependent since a significant difference was observed with *E. coli* and *K. pneumoniae* but not with *S. aureus* and *E. faecalis*. The prospective analysis of clinical cloudy urines showed that the InoqulA (INO1) provided a statistically higher number of discrete colonies than the WASP and manual inoculation resulting in a reduced time to report ID and AST results as well as reduced laboratory costs due to a decreased need to perform colony re-isolation. Finally, both the automated inoculation technology (magnetic bead versus loop) and the design of optimal streaking patterns had a significant impact on the performances of inoculation observed in this study.

This work represents one of the first studies conducted by an independent clinical diagnostic laboratory that demonstrates the true effectiveness of automated inoculation systems to generate isolated colonies positively impacting both the TAT and costs. Unlike manual inoculation, automated streaking systems are highly reproducible and offer the possibility to investigate new technical inoculation approaches to improve the quality and the quantification of colony growth and thus to further increase the productivity of the diagnostic laboratory.

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Figures Legends

Figure 1

Manual and automated semi-quantitative streaking protocols. Two manual quantitative plate inoculation patterns were performed by an experienced microbiologist with 10 µl loops following a zig-zag streaking pattern (MAN1) or a central single streaking throughout the plate followed by a zig-zag pattern (MAN2). Two similar automated quantitative plate inoculation patterns with the InoqulA BT and the WASP were performed following a zig-zag streaking pattern (INO1, WAS1) or a central single streaking of 20 mm followed by a zig-zag pattern (INO2, WAS2). The InoqulA INO1 pattern and the WASP WAS2 pattern were used as optimized factory designed semi-quantitative inoculation protocols. The manual MAN2 streaking approach was chosen as the conventional semi-quantitative manual inoculation used in most diagnostic laboratories. The INO2, MAN1 and WAS1 were chosen to use similar inoculation protocols allowing direct comparison between the different automated and manual systems.

Figure 2

Image analysis procedure. Image analysis was performed in 5 steps (A to E). (A) Raw image of the Petri-dish. (B) Surface pixels of the Petri-dish. (C) Pixels considered as growth. (D) Discrete colonies. (E) Four distinct clusters produced by Linear Discriminant Analysis. Each colour represents a different bacterial species.

Figure 3

Performance of manual, InoqulA and WASP plate inoculations at different bacterial concentrations of *E. coli*. Box plot of the number of discrete colonies following InoqulA (INO1, INO2), manual (MAN1, MAN2) and WASP (WAS1, WAS2) plate inoculations of different bacterial concentrations of *E. coli* ranging from 10^3 to 10^8 CFU/ml.

Figure 4

Performance of manual, InoqulA and WASP following streaking of monomicrobial samples at a concentration of 10^8 CFU/ml. Box plot of the number of discrete colonies of *E. coli* (ECOL), *E. faecalis* (EFEC), *K. pneumoniae* (KPN), and *S. aureus* (SAUR) following InoqulA (INO1, INO2), manual (MAN1, MAN2) and WASP (WAS1, WAS2) plate inoculations.

Figure 5

Recovery of discrete colonies of each bacterial species contained in polymicrobial samples following manual and automated inoculation. Box plot (A) and plate images (B) of the number of discrete colonies following InoqulA (INO1, INO2), manual (MAN1, MAN2) and WASP (WAS1, WAS2) plate inoculations of a polymicrobial sample containing *E. faecalis* at 10^7 CFU/ml, *S. aureus* at 10^6 CFU/ml, *E. coli* at 10^5 CFU/ml and *K. pneumoniae* at 10^4 CFU/ml representing a 1:1, 10:1, 100:1 and 1000:1 ratio between the highest and the lowest bacterial concentrations, respectively.

Figure 6

Performance of manual and automated inoculation on clinical urine samples. Yield of discrete colonies from 41 cloudy urines clinical samples positive for *E. coli* obtained following inoculation of 10 µl on chromogenic agar with the InoqulA (INO1, INO2), manually (MAN1, MAN2) and with the WASP (WAS1, WAS2) . Statistical higher number of discrete colonies (One way ANOVA multiple comparison, $p < 0.05$) was observed between the INO1 and the MAN1, MAN2, WAS1, WAS2 inoculations.

Figure 7

Impact of the performance of the different manual (MAN1, MAN2) and automated inoculation InoqulA (INO1, INO2) and WASP (WAS1, WAS2) systems on the time-to-report results and laboratory costs. (A) One discrete colony was required to perform identification by MALDI-TOF at day 1 post-inoculation. Re-isolation was performed when at least one colony was not obtained leading to a delayed time to report results of 1 working day (ID report at day 2). An additional laboratory cost of 5.8 EUR per re-isolation was calculated for each subculture and the results were extrapolated to 100 samples for clarity. (B) A minimum number of 6 discrete colonies grown on BBL chromogenic agar was required (1) to perform an ID by MALDI-TOF and (2) to make a bacterial suspension in 2 ml saline solution equivalent to a 0.5 McFarland turbidity to complete an AST at day 1 and to report the results at day 2. Thus, each sample containing less than 6 colonies needed re-isolation leading to a delayed time to report AST results of 1 working day (AST report at day 3). Similar to identification, an additional laboratory cost of 5.8 EUR per re-isolation was calculated for each subculture and the results were extrapolated to 100 samples for simplicity.

618

Table

Table 1. One way ANOVA multiple comparisons of the number of discrete colonies from cloudy urine samples obtained with the InoqulA (INO1, INO2), manually (MAN1, MAN2) and with the WASP (WAS1, WAS2)

Dunn's multiple comparisons test	Statistically Significant	P Values
INO1 vs. INO2	No	0.0993
INO1 vs. MAN1	Yes	< 0.0001
INO1 vs. MAN2	Yes	< 0.0001
INO1 vs. WAS1	Yes	< 0.0001
INO1 vs. WAS2	Yes	< 0.0001
INO2 vs. MAN1	No	0.0908
INO2 vs. MAN2	Yes	0.0010
INO2 vs. WAS1	No	0.9446
INO2 vs. WAS2	No	0.5419
MAN1 vs. MAN2	No	> 0.9999
MAN1 vs. WAS1	No	> 0.9999
MAN1 vs. WAS2	No	> 0.9999
MAN2 vs. WAS1	No	0.5038
MAN2 vs. WAS2	No	0.8836
WAS1 vs. WAS2	No	> 0.9999

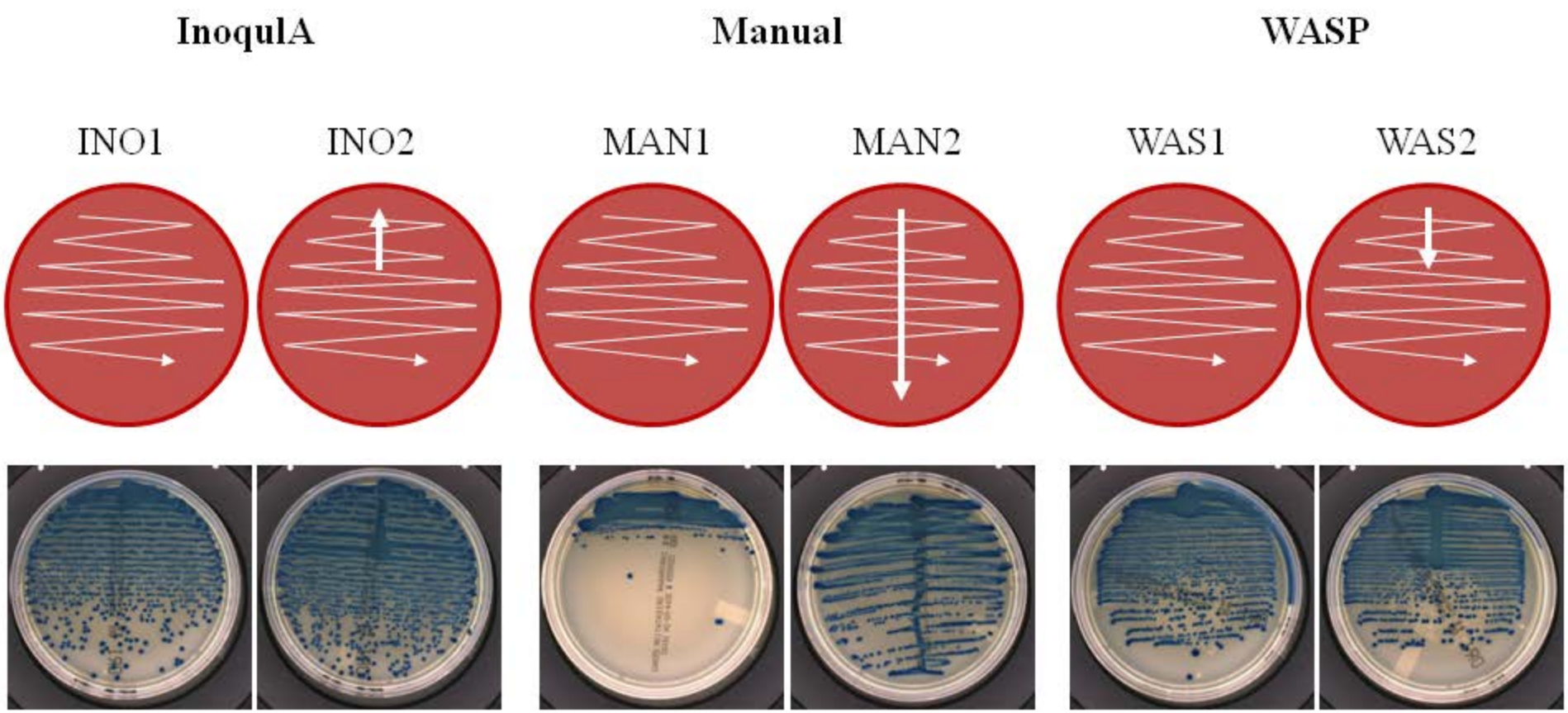

Figure 1

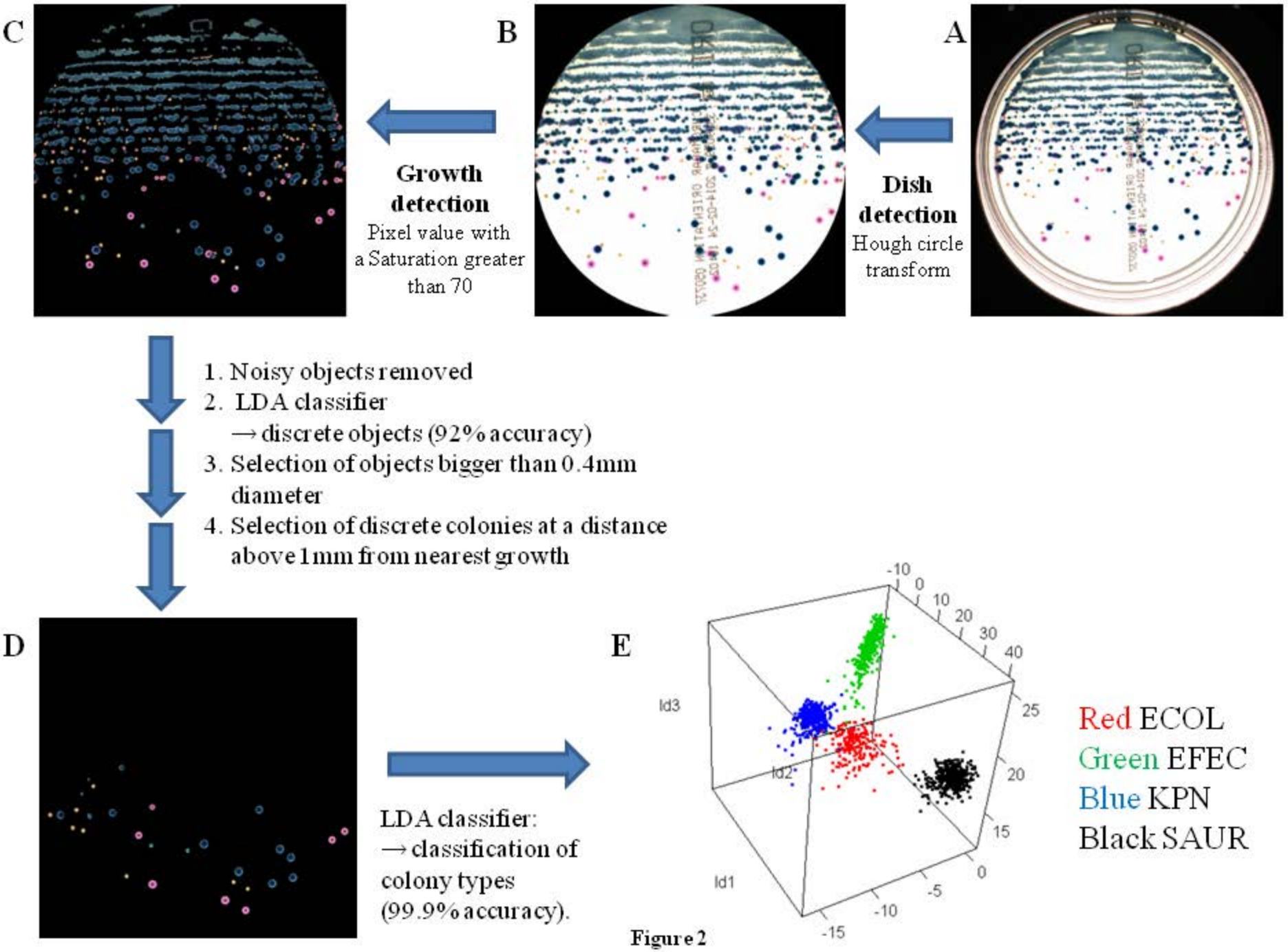
A
B
C
Dish detection
Hough circle transform
Growth detection
Pixel value with a Saturation greater than 70
1. Noisy objects removed
2. LDA classifier
→ discrete objects (92% accuracy)
3. Selection of objects bigger than 0.4mm diameter
4. Selection of discrete colonies at a distance above 1mm from nearest growth
D
LDA classifier:
→ classification of colony types
(99.9% accuracy).
E
ld1
ld2
ld3
Red ECOL
Green EFEC
Blue KPN
Black SAUR

Figure 2

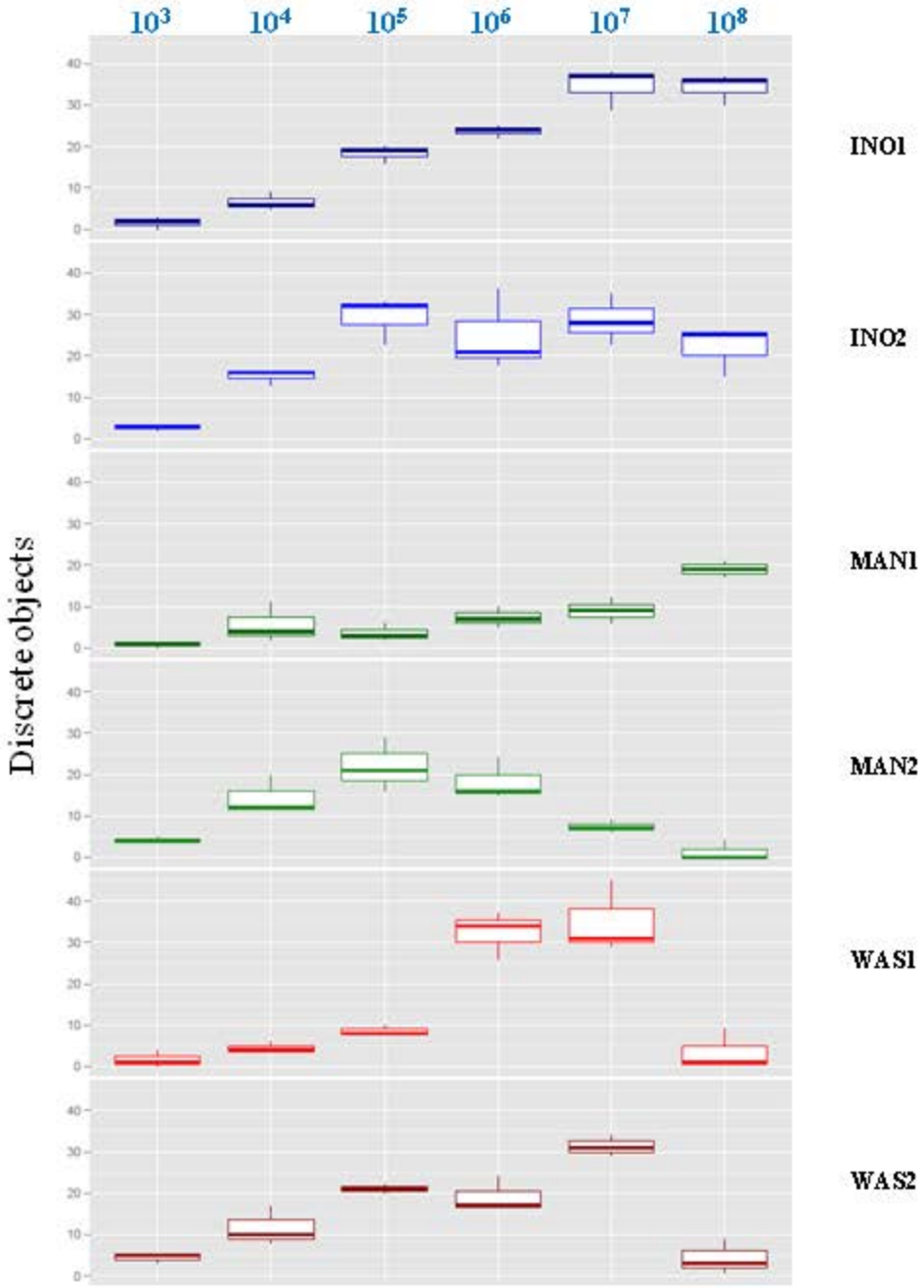
10^3
10^4
10^5
10^6
10^7
10^8
Discrete objects
INO1
INO2
MAN1
MAN2
WAS1
WAS2

Figure 3

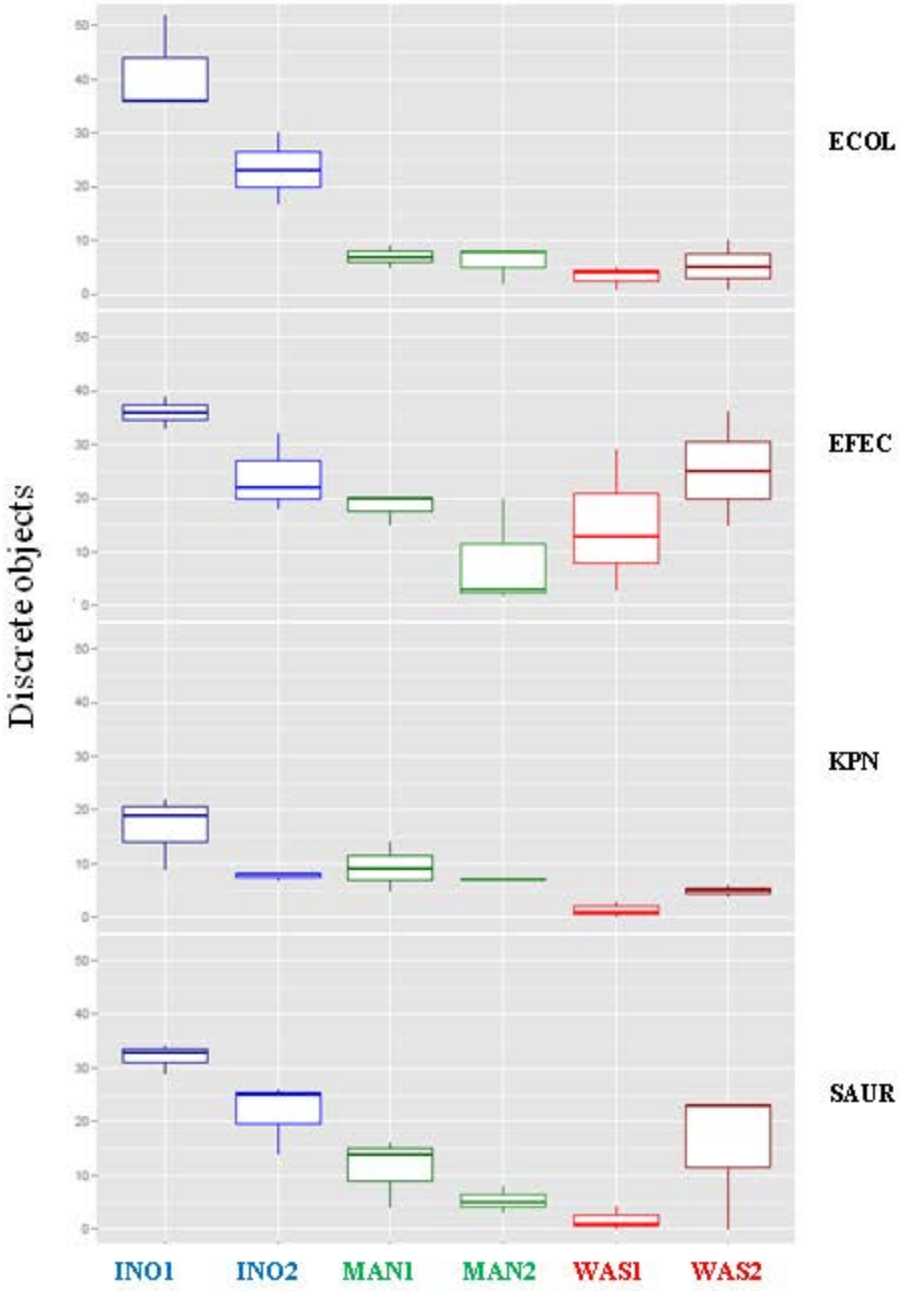
Discrete objects
ECOL
EFEC
KPN
SAUR
INO1
INO2
MAN1
MAN2
WAS1
WAS2

Figure 4

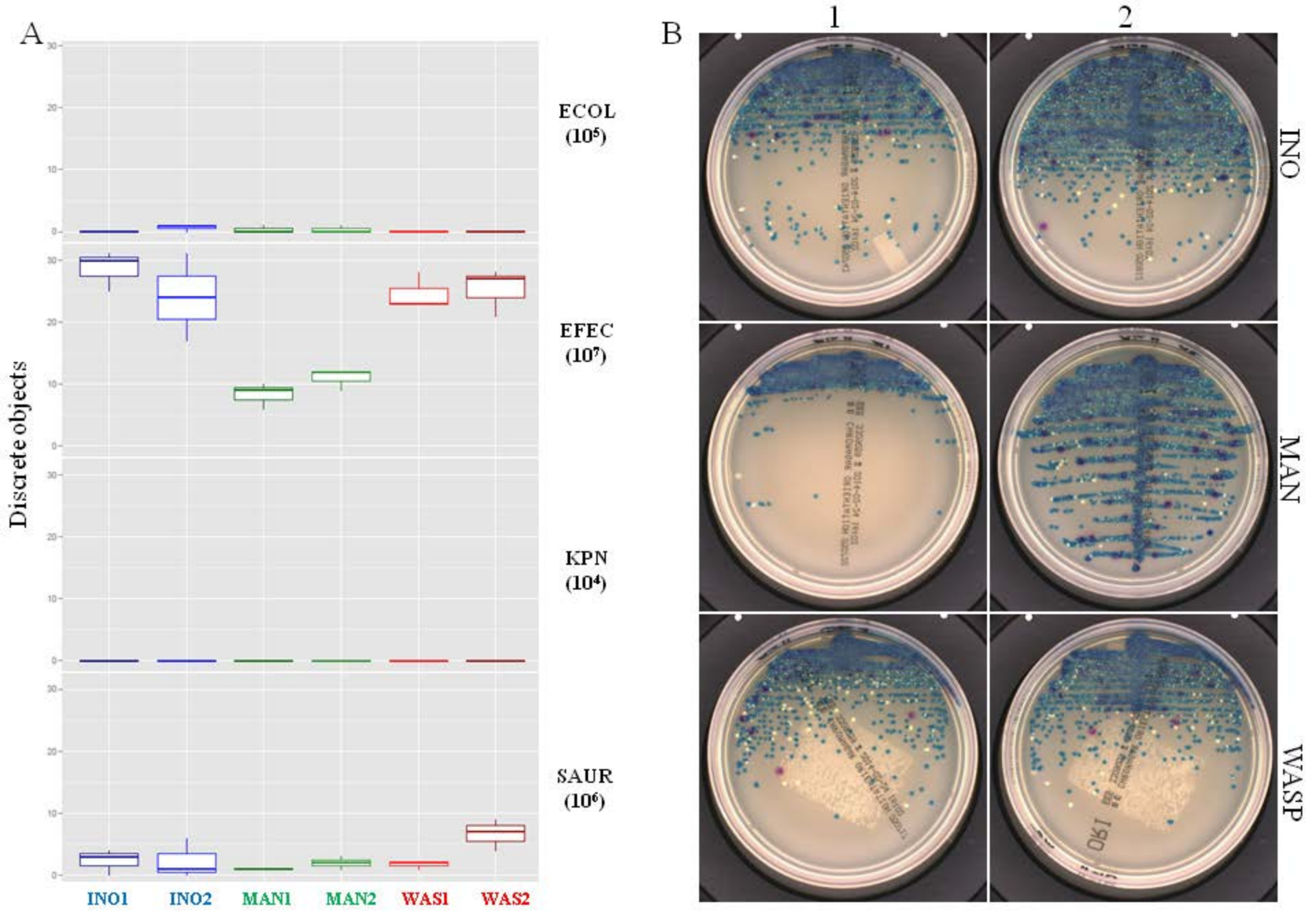
A
Discrete objects
ECOL
(10⁵)
EFEC
(10⁷)
KPN
(10⁴)
SAUR
(10⁶)
INO1
INO2
MAN1
MAN2
WAS1
WAS2
B
1
2
INO
MAN
WASP

Figure 5

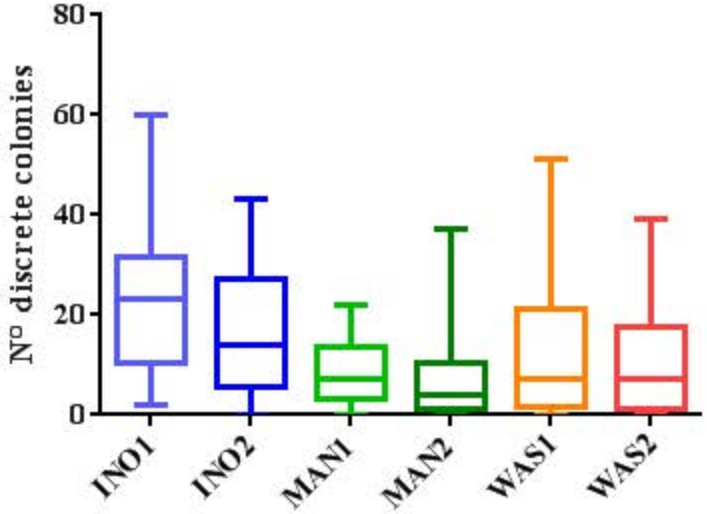
80
60
40
20
0
N° discrete colonies
INO1
INO2
MAN1
MAN2
WAS1
WAS2

Figure 6

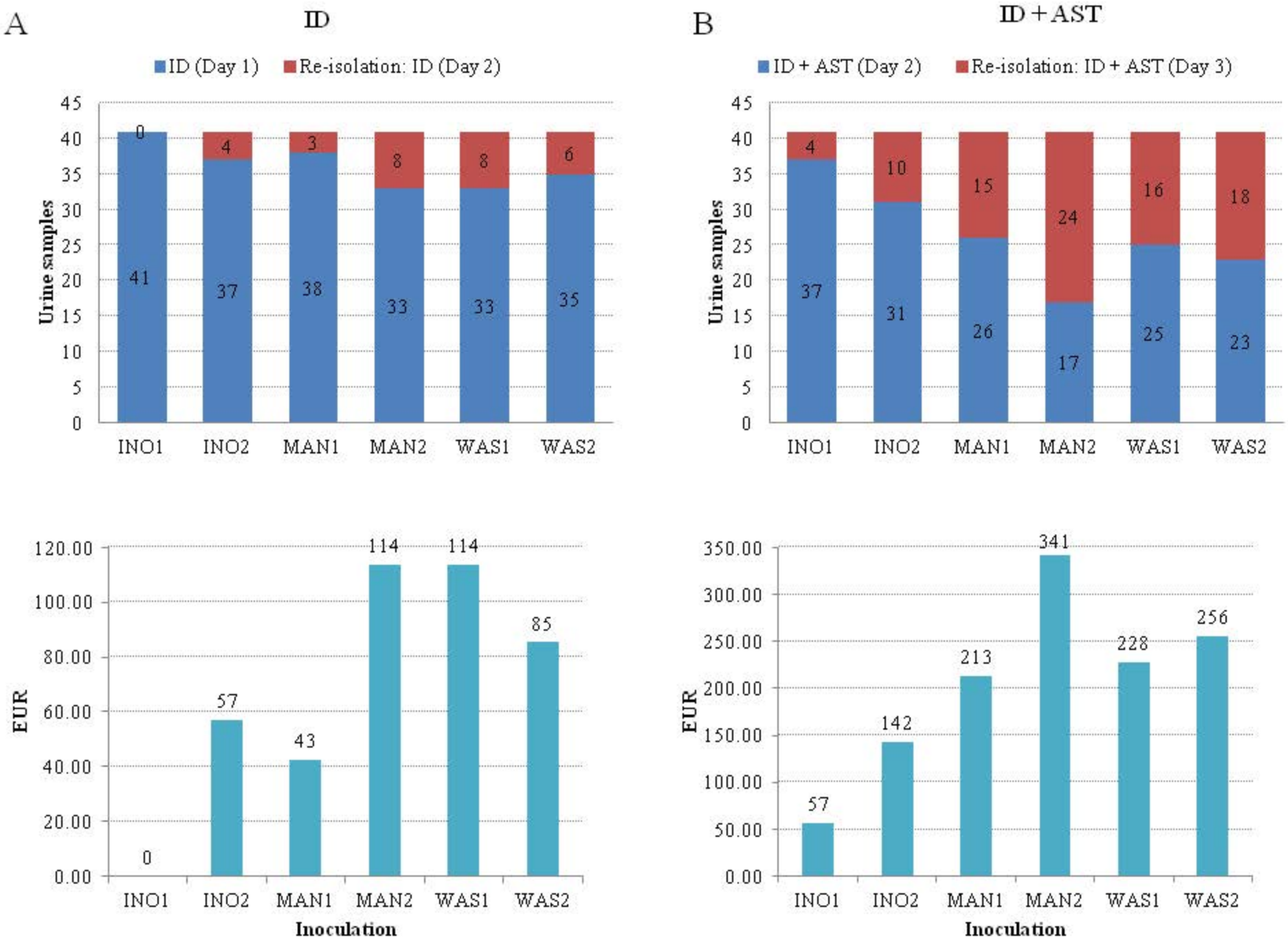
A
ID
ID (Day 1)
Re-isolation: ID (Day 2)
Urine samples
INO1
INO2
MAN1
MAN2
WAS1
WAS2
0
4
3
8
8
6
41
37
38
33
33
35
B
ID + AST
ID + AST (Day 2)
Re-isolation: ID + AST (Day 3)
4
10
15
24
16
18
37
31
26
17
25
23
EUR
Inoculation
0
57
43
114
114
85
57
142
213
341
228
256

Figure 7