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

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21 # Abstract

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

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

43

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

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

76 # Materials AND methods

77 ## Strains, media and bacterial suspension

78 *Escherichia coli* strain ATCC 25922, *Klebsiella pneumoniae* strain ATCC BAA-1706,
79 *Staphylococcus aureus* strain ATCC 29213 and *Enterococcus faecalis* strain ATCC 29212 were
80 grown on Columbia agar with 5% sheep blood (BD columbia III agar, BD, Franklin Lakes, NJ,
81 USA) at 37°C in 5% CO_2 atmosphere incubators. Colonies of each bacterial species were
82 utilized to prepare a bacterial suspension in saline solution adjusted to a 0.5 McFarland turbidity
83 measured with a Densitometer Densicheck instrument (Biomérieux, Marcy-l'Etoile, France) and
84 corresponding to a bacterial concentration of 10^8 colony forming units per ml (CFU/ml).
85 Different concentrations of monomicrobial suspension in saline solution ranging from 10^8 to 10^3
86 CFU/ml were prepared by doing serial 10-fold dilutions in saline solution. All bacterial
87 suspensions were plated on Columbia agar with 5% sheep blood to verify the number of
88 CFU/ml. Polymicrobial suspensions containing 4 bacterial species at different ratio ranging from
89 1:1 to 1:1000 (supplementary table 1) were obtained by mixing different concentrations of the
90 diluted and non-diluted monomicrobial suspensions.

91 ## Sample collection

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

98 ## Inoculation and incubation

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

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

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

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

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

131 **Analysis of reporting times and laboratory costs**

132 The time to report results and laboratory costs were calculated based on the ability of the
133 different systems to produce a minimal number of isolated colonies to perform identification by
134 MALDI-TOF and AST. The minimal number of *E. coli* colonies grown on chromogenic BBL
135 CHROMagar Orientation agar plates in a normal ambient atmosphere for 20 hours at 35°C was
136 determined following conventional laboratory procedures with a minimum of 1 discrete colony
137 required for ID by MALDI-TOF and a minimum of 5 colonies required to make a 2ml bacterial
138 suspension in saline solution with a turbidity of 0.5 McFarland for AST. A delayed time to report
139 result of 1 working day (16hr to 24 hr) and additional laboratory costs were applied when the
140 minimal number of isolated colonies required to perform the ID and AST procedures was not
141 obtained. The laboratory cost per re-isolation was calculated in both Swiss Francs (CHF) and
142 European Euros (EUR) based on consumable prices and labor costs including social security
143 charges applied at the university hospital of Lausanne, Switzerland, as follows: Agar plate (1
144 CHF / 0.8 EUR), plastic loop (0.1 CHF / 0.1 EUR), 5 min working time to perform a re-isolation
145 (5.9 CHF / 4.9 EUR) for a total of 7 CHF or 5.8 EUR per re-isolation. The conversion rate of 1
146 EUR = 1.2 CHF was calculated in November 2014 and may be subjected to variations due to the
147 volatility of the foreign exchange rate. The experimental working time of 5 min to perform re-
148 isolation includes the following tasks: (1) Collect the agar plate containing the sample to re-
149 isolate in the incubator, (2) collect a sterile plate for subculture in the cold room (3) collect a
150 plastic loop, (4) plate labelling, (5) colony picking and 4 quadrant plate streaking, (6) store the
151 plates into the incubators. The working time of 5 min was measured and used for an
152 experimental modelization of additional laboratory costs due to the requirement of subculture to
153 perform ID and AST from discrete colonies in both automated and conventional laboratories.
154 The measured working time strongly depends on the organisation of the laboratory workflow and
155 may greatly vary between laboratories.

156 **Imaging and Image analysis**

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

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

169 The Hough circle transform (10) was used as a robust method giving the correct position of the
170 Petri-dish for every image in the set (figure 2A and 2B). Because of refractions and reflections at
171 the dish border, a few millimetres of the outer border of the Petri-dish image were ignored by the
172 image analysis software to increase measurement accuracy. The size of this border is equal
173 among all images preventing any bias towards any image (figure 2B). Pixels with a high
174 saturation value of 70 were considered as non-white and were selected as object pixels (figure
175 2C). A lower value resulted in more growth pixels around each colony while a higher value
176 resulted in less growth. As a result, a white agar background was required for a reliable detection
177 of bacterial growth.

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

185 Classification of discreteness and bacterial species was done by a Linear Discriminant Analysis
186 (LDA) (11, 12) based classifier. LDA is a linear model which uses statistics of the data to
187 determine the optimal separation between the different classes. A data set of 3379 images of
188 discrete and non-discrete colonies of *E. coli*, *K. pneumoniae*, *S. aureus* and *E. faecalis* were
189 defined by a technician resulting in the defined data set. The LDA classifier was trained with
190 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

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

Statistical analysis

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

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

232 # Results

233 ## Image analysis

234 The image analysis was performed in 5 steps as shown in figure 2. First, the Petri-dish surface
235 was determined by using a Hough circle transform (10) which was specifically suited for
236 detecting circles (figure 2B). Secondly, bacterial growth was determined by selecting pixels with
237 high colour saturation (e.g. if the colour is different from white) (figure 2C) (13). Connected
238 pixels were grouped into objects and objects smaller than 0.4 mm were considered as noise and
239 were removed. Consequently, a group of connected pixels with high colour saturation were
240 considered as growth and called an object. Then, each object could either be one discrete colony
241 or several connected colonies. Discrete objects were recognized by the discreteness LDA
242 classifier. Objects with a diameter below 0.4 mm and/or with a distance to the nearest growth
243 below 1 mm were removed. All remaining objects were considered as discrete colonies (figure
244 2D). Finally, the bacterial species of each discrete colony was determined by a bacterial species
245 LDA classifier trained with four bacterial species, *Escherichia coli*, *Klebsiella pneumoniae*,
246 *Enterococcus faecalis* and *Staphylococcus aureus* (figure 2E). Consequently, in this study, only
247 these four bacterial species could be automatically recognized on the agar plates.

248 An evaluation of the accuracy to correctly classify discrete and non-discrete objects as well as
249 bacterial colony species was performed for each step involved in the image analysis process
250 (figure 2C to 2E). The evaluation was performed using a defined data set containing 3379 images
251 of known objects belonging to discreteness (discrete, non-discrete) and bacterial species classes
252 (1915 Non-discrete objects, 423 *Escherichi coli*, 353 *Klebsiella pneumoniae*, 199 *Enteroccocus*
253 *faecalis* and 489 *Staphylococcus aureus*). Objects from all the bacterial species classes were
254 discrete. The defined data set was divided in a training set and an evaluation set to be used for a
255 two-fold cross validation.

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

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

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

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

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

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

292 discrete colonies at different bacterial concentrations whereas the MAN2, WAS1 and WAS2 293 inoculation methods were characterized by an increased yield of discrete colonies followed by a 294 significant reduced performance when reaching moderate (10^6/ml with the MAN2) to high 295 bacterial concentrations (10^7/ml with the WAS1 and WAS2 inoculations), respectively. The 296 automated inoculation systems InoqulA and WASP showed a statistically significant higher yield 297 of discrete colonies ($p < 0.05$, multiple comparisons of means) than manual inoculation at 10^7 298 CFU/ml whereas the InoqulA produced statistically more discrete colonies ($p < 0.05$, multiple 299 comparisons of means) than the WASP and manual inoculation at 10^8 CFU/ml (supplementary 300 table 2).

301

302 **Quality of isolation of defined monomicrobial samples**

303 As demonstrated with the inoculation of different bacterial concentrations of *E. coli* (figure 3), a 304 significant difference between the inoculation systems was mainly observed at bacterial 305 concentrations $\geq 10^7$ CFU/ml. The streaking quality of manual and automated inoculation was 306 thus assessed by measuring the yield of discrete colonies following inoculation of four bacterial 307 species suspensions at a concentration of 10^8 CFU/ml. Two Gram negative and two Gram 308 positive bacteria, *Escherichia coli, Klebsiella pneumoniae, Staphyloccocus aureus* and 309 *Enterococcus faecalis*, were used to integrate morphological and physiological traits differences 310 that may impact the streaking efficiency of the manual and automated systems. In addition, 311 colonies of these 4 bacterial species growing on chromogenic agar exhibit different colours that 312 facilitate the recognition and classification of discrete colonies by the Vision Lab v3.43 313 software. The yield of discrete colonies and the differences observed between the automated and 314 manual inoculations were bacterial species dependent (figure 4). All the streaking methods 315 except MAN2 were able to produce a high yield of discrete colonies of *E. faecalis*. However, the 316 INO1 inoculation produced a statistically higher number of discrete colonies ($p < 0.05$, multiple 317 comparisons of means) than manual and WAS1 inoculations (supplementary table 3). To the 318 contrary, a lower yield of *K. pneumoniae* isolated colonies was obtained with the 6 streaking 319 approaches compared to the other bacterial species, with no statistical difference between 320 automated and manual inoculations (supplementary table 3). The yield of discrete colonies of *E.* 321 *coli* and to a lesser extend of *S. aureus* was strongly dependent on the streaking method. A 322 statistically significant higher yield of *E. coli* discrete colonies ($p < 0.05$, multiple comparisons

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

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

342 **Quality of isolation of defined polymicrobial samples**

343 The ability of the different inoculation systems to obtain discrete colonies of each bacterial
344 species contained in a polymicrobial sample was assessed to determinate their discriminative
345 power. Eleven polymicrobial suspensions containing *E.coli*, *K. pneumoniae*, *S. aureus* and *E.*
346 *faecalis* were obtained by mixing the 4 bacterial species at different ratio ranging from 1:1 to
347 1000:1 between the highest and lowest bacterial concentrations (supplementary table 1). The
348 results obtained with mixes M01 to M10 (supplementary figure 3) were similar to those observed
349 in the polymicrobial suspensions mix M11 (figure 5). The mix M11 was composed of *E. faecalis*
350 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
351 CFU/ml. The InoqulA and the WASP inoculation produced a statistically significant higher yield
352 of *E. faecalis* discrete colonies ($p < 0.05$, multiple comparisons of means) compared to manual

353 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.

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

364 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

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

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

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

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

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

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

521

522

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559 # Figures Legends

560 **Figure 1**

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

573 **Figure 2**

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

578 **Figure 3**

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

583 **Figure 4**

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

588 **Figure 5**

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

596 **Figure 6**

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

603 **Figure 7**

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

618

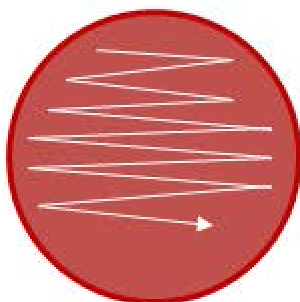
Table

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)

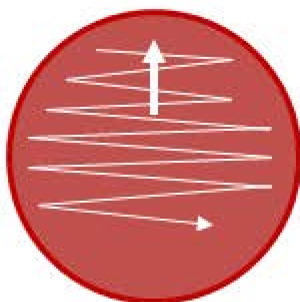
Dunn's multiple comparisons test	Statistically Significant	P Values
INO1 vs. INO2	No	0.0993
INO1 vs. MAN1	Yes	< 0.0001
INO1 vs. MAN2	Yes	< 0.0001
INO1 vs. WAS1	Yes	< 0.0001
INO1 vs. WAS2	Yes	< 0.0001
INO2 vs. MAN1	No	0.0908
INO2 vs. MAN2	Yes	0.0010
INO2 vs. WAS1	No	0.9446
INO2 vs. WAS2	No	0.5419
MAN1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

InoqulA | **Manual** | **WASP**

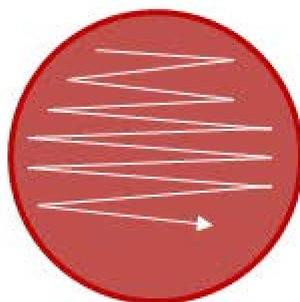
INO1



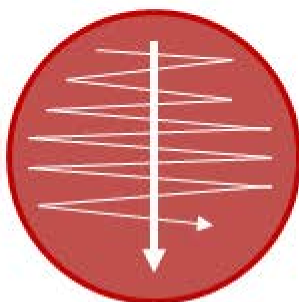
INO2



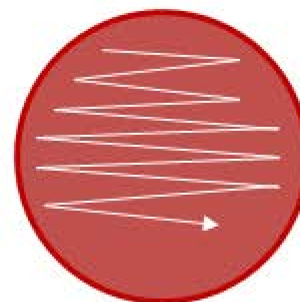
MAN1



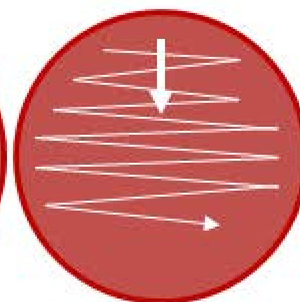
MAN2

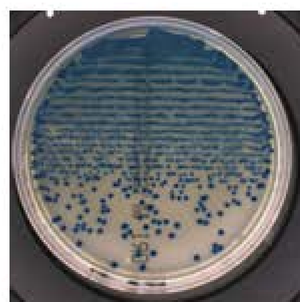


WAS1



WAS2





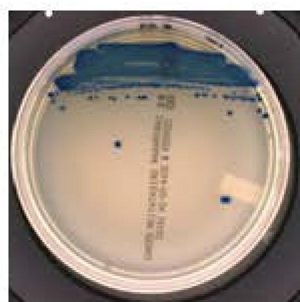
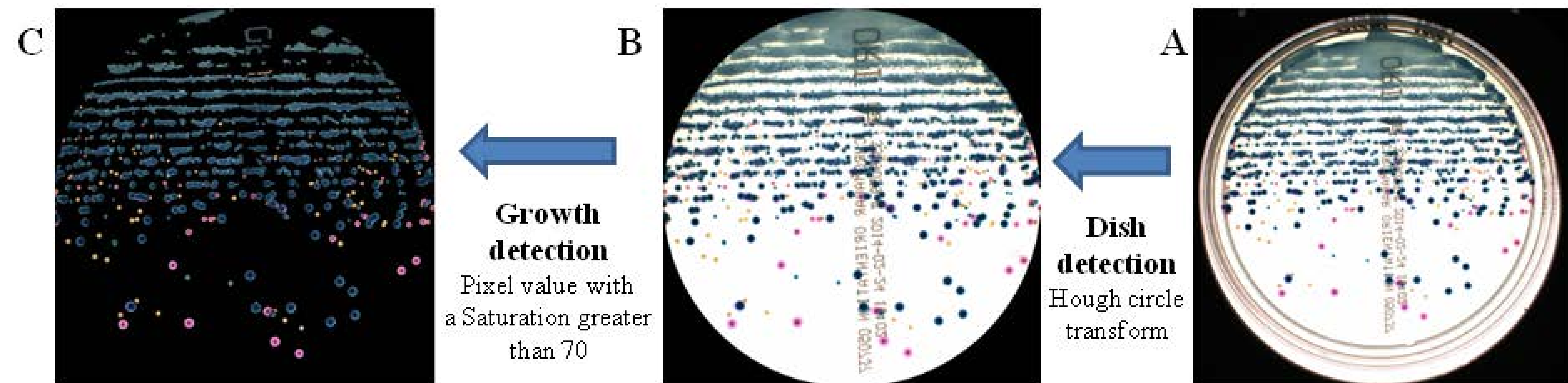
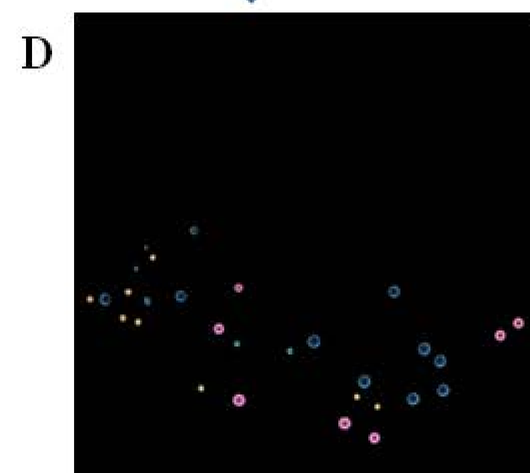


Figure 1



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



LDA classifier:
→ classification of colony types (99.9% accuracy).

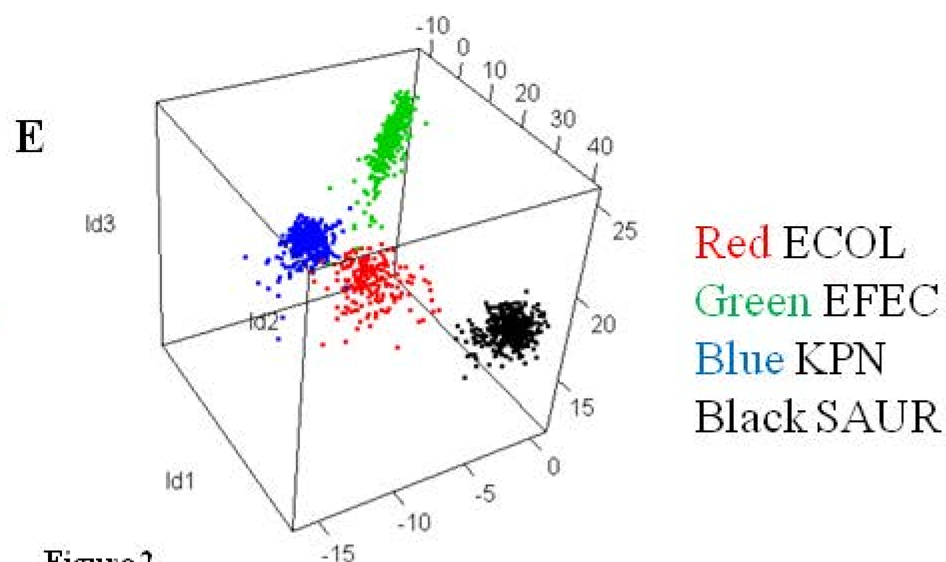


Figure 2

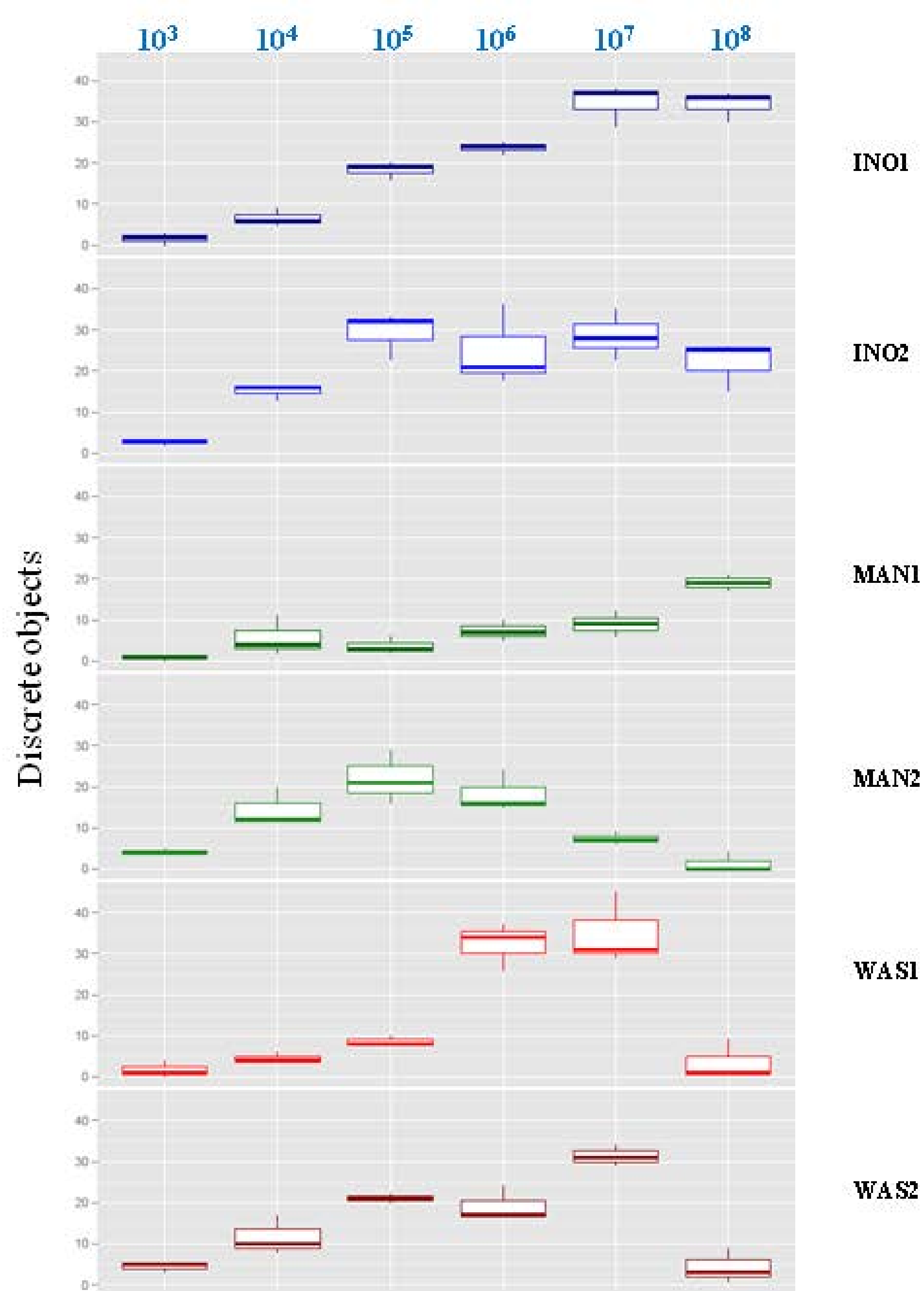


Figure 3

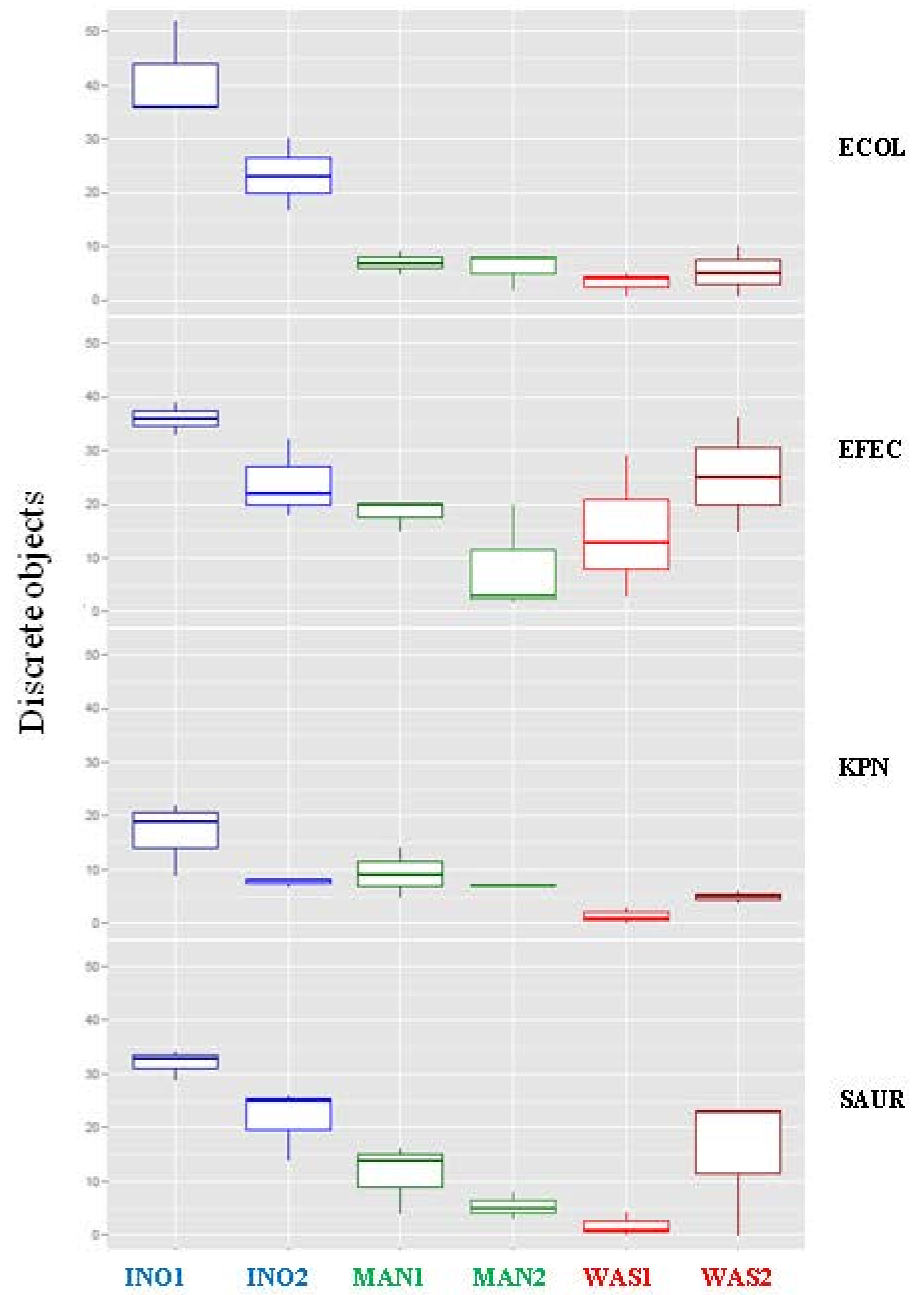


Figure 4

A

Discrete objects

ECOL (10^5)

EFEC (10^7)

KPN (10^4)

SAUR (10^6)

INO1 INO2 MAN1 MAN2 WAS1 WAS2

B

1 2

INO

MAN

WASP

Figure 5

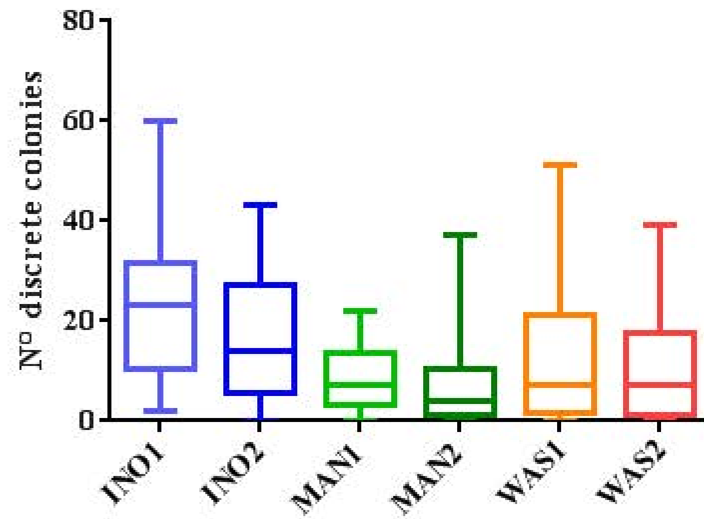
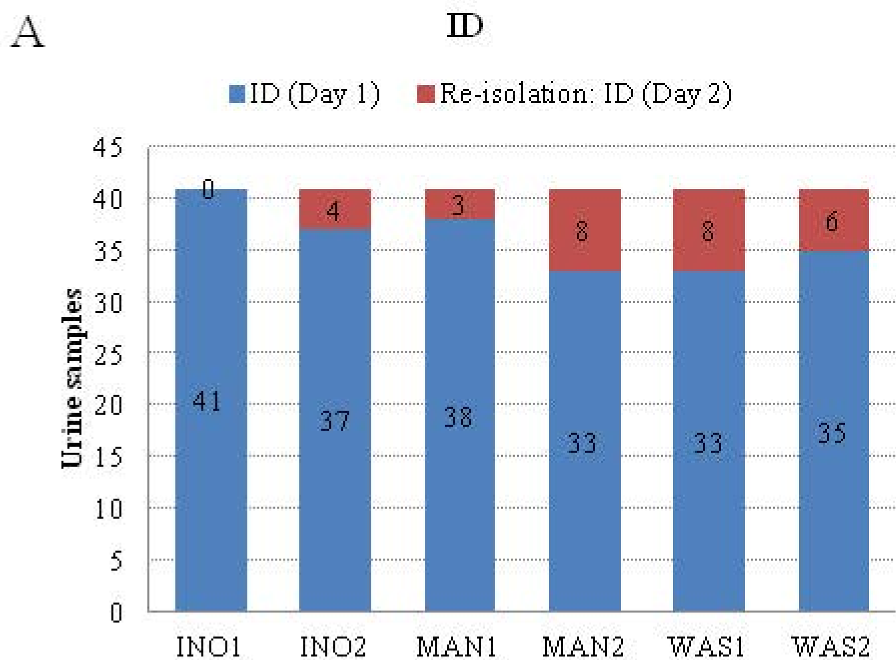
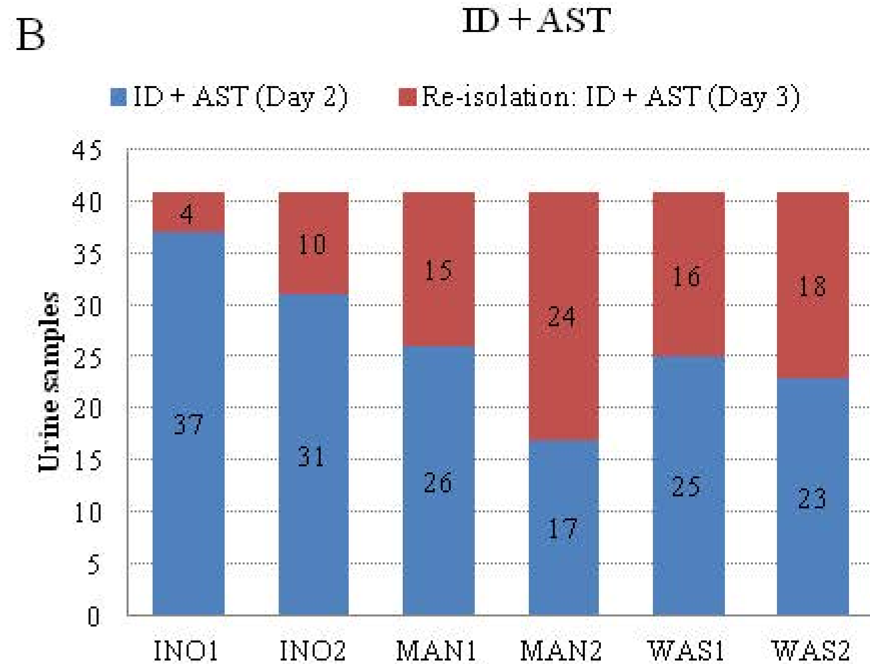
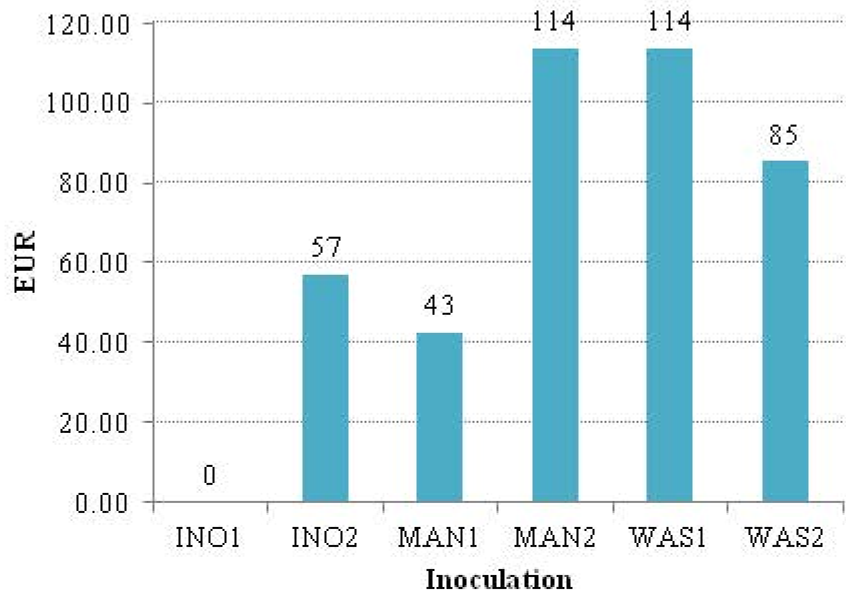


Figure 6







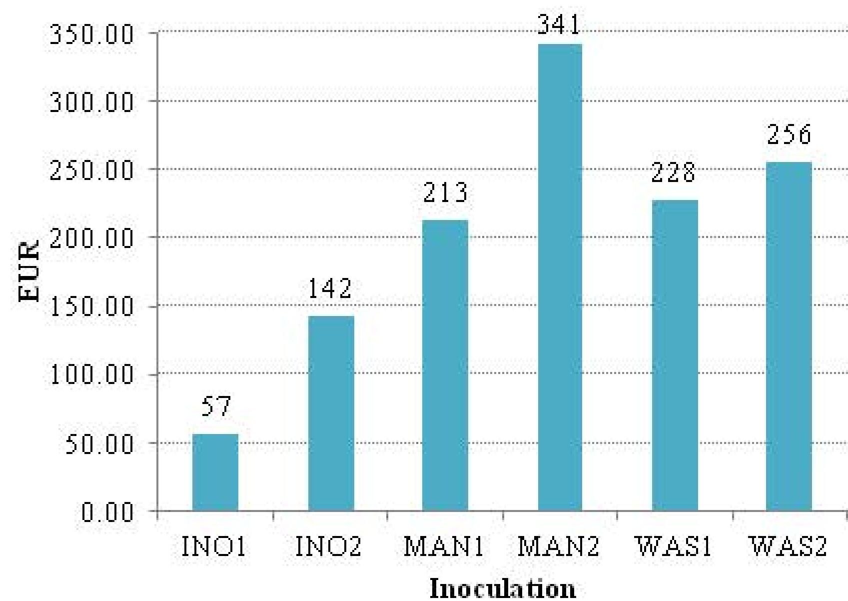


Figure 7