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1 **Impact of freeze/thaw cycles and single freezing at -80°C on the viability of aerobic**
2 **bacteria from rectal swabs performed with the ESwab™ system**

3 Rindala Saliba¹, Jean Ralph Zahar^{1, 2}, Fatma El Allaoui¹, Etienne Carbonnelle ^{1, 2} and
4 Mathilde Lescat^{1, 2, *}

5 ¹APHP, Hôpitaux Universitaires de Paris Seine Saint-Denis, Bondy, France

6 ² Université Paris 13, Université Paris Diderot, INSERM, IAME, UMR 1137, Sorbonne Paris
7 Cité, Paris, France

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9 **Running title:** bacteria viability ESwab™

10 **Keywords:** *Escherichia coli*, aerobic bacteria, ESwab™, freezing thawing, survival, impact
11 for strain collection

12 * Corresponding author

13

14 **Abstract**

15 The testing of bacterial preservation should be included in preliminary studies to
16 epidemiological studies. In the case of multidrug-resistant organism (MDRO) studies,
17 quantifications of the bacteria makes it possible to understand their emergence. The purpose
18 of this preliminary study was to evaluate the performance of ESwab™ on survival of
19 *Escherichia coli*, *Klebsiella pneumoniae* and *Enterococcus faecalis*, based on the number of
20 freezing and thawing (F/T) cycles at -80 ° C and freezing time. A first experiment with 9

21 samples showed that multiple F/T cycles drastically affected *Enterobacteriaceae* viabilities
22 and less *E. faecalis* one. A single freezing maintained the three species viabilities during three
23 weeks. A second experiment showed that *E. coli* survival was maintained with a 3-month
24 single freezing. This study which used a limited number of bacterial isolates is however a
25 proof of concept establishing the utility of ESwab™ samples when freezed once in
26 quantitative studies of bacteria.

27 **Introduction:**

28 Understanding the basics of the emergence of multidrug-resistant organisms (MDROs)
29 has become a critical challenge in recent decades. *Enterobacteriaceae* are among the most
30 common MDROs in the world and are generally carried by patients at the commensal level
31 before being involved in pathologies (and thus isolated in clinical samples). *Escherichia coli*
32 can be considered as one of the most dangerous MDRO because it is the most effective
33 human intestinal commensal and becomes, depending on factors related to the host or the
34 strain, a formidable pathogen (Tenailon et al. 2010). In addition, extended-spectrum β -
35 lactamase (ESBL) -based isolates mainly belong nowadays to this species, both in hospitals
36 and communities, and constitute a major public health problem worldwide (Woerther et al.
37 2013). Moreover, a high relative abundance of ESBL *E. coli* has been associated with longer
38 fecal carrying time and higher risk of infection (Ruppé et al. 2013). Many *in vivo* and *in vitro*
39 studies tried to identify the basis of commensalism which is the natural behavior of *E. coli*
40 isolates whether it is resistant or not (Lescat et al. 2017). The other MDROs belong mainly to
41 the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*,
42 *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (Boucher et
43 al. 2009). These species are also opportunistic pathogens, thus quantitative studies of their
44 prevalence in the digestive gut are essential. (Yamamoto et al. 1997; Ruppé et al. 2013; de

45 Lastours et al. 2016).

46 Several steps in the analyses of bacteria from gastrointestinal tract are crucial and are
47 directly related to a good diagnostic performance in Clinical Microbiology laboratories. Thus,
48 whatever the method used (molecular or culturomics), it is essential that the sampling,
49 transport and storage procedures do not alter the microbial composition (Tedjo et al. 2015).
50 The best results are obtained when the viability of the microorganisms is maintained, allowing
51 a preservation of the relative proportions of all the microorganisms present in the clinical
52 samples between them (Van Horn and Rankin 2007) (Nys et al. 2010). However, studies
53 evaluating the stability of bacteria in these transport devices have shown that it does not
54 exceed several days. The transport device, ESwab™, contains a flocced nylon swab and a
55 modified "Amies" transport liquid. ESwab™ is a system with many benefits, including
56 increased uptake and release of bacteria with flocced nylon, increased bacterial survival in the
57 transport fluid, and easy-to-use system suitable for automation. Samples taken for bacterial
58 testing, which can not be delivered immediately to the laboratory, may be refrigerated (4-8°C)
59 or stored at room temperature (20-25°C) for treatment within 48 or 24 hours after collection,
60 respectively (Nys et al. 2010). According to the manufacturer, ESwab™ liquid could be
61 frozen at -20 ° C; however, after thawing, only molecular bacterial tests can be performed
62 (Human and Jones 2004). Indeed, Hirvonen *et al* performed cultures of frozen ESwab™
63 liquids containing enteric pathogens during two weeks at -20°C and 3 months at -70°C and
64 were not able to observe bacteria (Hirvonen and Kaukoranta 2014). However, this study was
65 performed to evaluate the viability of enteric pathogens (*Salmonella spp*, *Shigella sp* and
66 others) and this is the only one study we found on the subject. Nowadays, numerous labs use
67 ESwab™ system to sample the patients. In consequence, evaluating this device in quantitative
68 studies could be very useful.

69 So far, quantitative studies of *E. coli* in the faeces were performed using fresh total
70 stools or rectal swabs promptly performed in the laboratory. These samples could be directly
71 plated on selective media or freezed at -80 ° C in brain heart infusion (BHI) supplemented
72 with glycerol (Yamamoto et al. 1997; Ruppé et al. 2013; de Lastours et al. 2016). These
73 procedures are time-consuming and can not be applied in many labs explaining the small
74 number of such studies. Quantitative studies on other bacteria are even rarer, based only on
75 the analysis of total microbiota. These data are obtained using molecular analysis and may not
76 be precise or may not reflect viable bacteria. This shows the need for other precise
77 quantitative cultures (Dautzenberg et al. 2016; Shimasaki et al. 2018). Thus, having the
78 possibility of freezing these devices directly could be useful and increase the feasibility of
79 such experiments. Pretesting of procedures for specimen collection, transport and
80 conservation should be part of preliminary studies for trials. The aim of this preliminary
81 study, was (i) to evaluate the survival of aerobic bacteria from rectal swab placed in the
82 transport medium ESwab™ according to the number of freezing at -80°C and thawing (F/T)
83 cycles and (ii) the specific survival of *E. coli* isolates from rectal swab placed in the transport
84 medium ESwab™ during a long period (3 months).

85 **Materials and Methods:**

86 The survival of aerobic bacteria in the ESwab™ (Copen Diagnostics, Italy) devices
87 was investigated by quantification of aerobic bacteria during two experimentations. Both
88 experiments were performed using samples of clinical rectal ESwab™, chosen randomly, in
89 the laboratory of Microbiology of Jean Verdier Hospital, Bondy, France.

90 In the first experiment, 9 samples were treated for 3 weeks by both methods A and B,
91 in order to compare the quantifications of each aerobic bacterium with their initial
92 quantification. In Method A, for each sample, a single 400 µL aliquot of the ESwab™

93 transport fluid containing the rectal swab underwent F/T cycles at each stage (at 1 week, 2
94 weeks, and 3 weeks). In Method B, for each sample, three 100 µL aliquots were initially
95 prepared from the ESwab™ transport fluid containing the rectal swab and then frozen at -80 °
96 C and used for quantification at each stage (meaning that each aliquot was thawed only once
97 at 1 week, 2 weeks and 3 weeks). Whatever the method, the quantification of aerobic bacteria
98 was performed using 100 µL of the transport fluid of ESwab™. The suspension was first
99 serially diluted in with in 10-fold dilutions until a dilution of 10⁻⁷ was achieved. Then, a 100
100 µl sample of each dilution was inoculated on UriSelect4 plates (Bio-Rad, La Coquette
101 Marches, France) for traditional quantitative bacterial culture, for a 18-24 hours incubation at
102 37°C without any supplemental in the atmosphere. Quantitation of each aerobic bacterium
103 was then obtained by the same laboratory technologist which performed visually each count
104 of aerobic bacteria colonies on three dilution plates. The relative quantification of each point
105 was obtained by dividing the result of each quantification of aerobic bacteria each time (1
106 week, 2 weeks and 3 weeks) by the initial result of each quantification of aerobic bacterium at
107 time 0 (TO) and multiplied by 100 and then expressed in percentage.

108 In the second experiment, 4 other samples containing only *E. coli* isolates were treated
109 during 3 months by method B (in which thawing and quantifications were performed every
110 month) for comparison over time after a first quantification of isolates of *E. coli*.

111 At the beginning of the study, a first identification of bacterial colonies was performed
112 on a panel of colonies that grew on UriSelect4 plates, allowing the identification at the species
113 level using the Microflex bench-top Matrix Assisted Laser Desorption Ionisation-Time of
114 Flight (MALDI-TOF) mass spectrometer (Bruker, Champs-sur-Marne, France, database:
115 MBT IVD library v 8.0). Then, we decided to interpret the identification of all other bacteria

116 with the same size, colour and aspect of the colonies after a first identification by MALDI-
117 TOF.

118 All statistics were computed performed using R software (R Development Core Team,
119 2009, Vienna Austria) and statistical significance was determined at a p-value of less than
120 0.05.

121 **Results**

122 In the first experiment, the survival of each aerobic bacterium in the ESwab™
123 transport liquid was evaluated by calculating a ratio between the number of aerobic bacterial
124 colonies over time and the initial number, using both methods A and B. Of the 9 samples, 6
125 were positive for the detection of *E. coli*, two for *K. pneumoniae* and eight for *E. faecalis* at
126 time 0 (T0). Once detected, all aerobic bacterial quantifications ranged from $2.0 \cdot 10^4$ and 1.5
127 10^7 colony forming units (CFU) / mL of ESwab™ fluid (Table 1). Variations were observed
128 between the two methods and the different bacteria. The relative quantifications of both
129 *Enterobacteriaceae* showed a drastic decrease when samples were subjected with less than
130 15% of recovery after 3 cycles of F/T (method A). In contrast, with a single freezing (method
131 B), the relative quantifications remained stable for both *Enterobacteriaceae*. Specifically, a
132 statistical comparison between the quantifications obtained in the two methods showed a
133 significantly higher survival of *E. coli* isolates with a single freezing (relative quantification at
134 100% throughout the experiment) ($p= 0.0002$ using a Wilcoxon test), while isolates of *K.*
135 *pneumoniae* were recovered at a relative quantification at 80% (we did not performed
136 statistical comparison due to the low number of isolates). *E. faecalis* relative quantifications
137 decreased to 60% when the samples were processed by method A (F/T cycles) while it
138 decreased to 80% with method B (single freezing) (this difference was not significant using a
139 Wilcoxon test). Finally, when we compared the relative quantifications of *E. coli* and *E.*

140 *faecalis* after F/T cycles (method A), we observed that *E. faecalis* isolates were more resistant
141 to F/T cycles (method A) ($p= 0.0149$ using a Wilcoxon test).

142 In the second experiment, four samples each divided in three aliquots of ESwab™
143 fluid containing exclusively *E. coli* isolates were frozen for three months to specifically
144 determine for this species, for a longer period, whether stability could be achieved. Each
145 aliquot was thawed every month and the relative quantification calculated. We observed that
146 *E. coli* viability was excellent when one F/T cycle was performed. The quantification of *E.*
147 *coli* isolates in the four samples presented a mean value of $2 \cdot 10^6$ CFU / mL (ranged from
148 1.010^5 to $5.0 \cdot 10^6$) at TO (Table 1) and was maintained over the course of three months.

149 Discussion and conclusion

150 In this study, we evaluated the survival of aerobic bacteria from rectal swabs placed in
151 the transport fluid ESwab™ after F/T cycles for a period of 3 weeks. In a second step, we
152 specifically studied the survival of *E. coli* isolates taken from a rectal swab placed in the
153 ESwab™ transport medium for a longer period (3 months).

154 It was not possible to weigh the feces collected on the rectal swabs because the
155 analyzes were performed from patient samples and swab weighing would have been
156 necessary before sampling, but was not possible in the clinical setting we used. The
157 comparison with a quantification per gram of feces is therefore not very precise.

158 The results of the first experiment suggest that F/T cycles significantly altered the
159 viability of enterobacterial species (significant results for *E. coli* isolates). *E. faecalis* isolates
160 are also decreased but can be recovered after 3 F/T cycles at 60% which is significantly
161 higher compared to *E. coli* isolate recoveries. When aliquots are performed initially and only
162 one thawing is performed all three species showed higher viabilities between (80 to 100% of

163 recovery). We have not been able to explain such divergences in bacterial families and we
164 have found only the study by Gao *et al* who compared the survival of *E. coli* and *E. faecalis*
165 isolates frozen in sterile water at different temperatures. They also observed increased
166 inactivation of all isolates proportional to the number of F/T cycles with higher survival of *E.*
167 *faecalis*. They did not observe higher survival of the *E. coli* species when only one freezing
168 was carried out, but the duration of freezing is not indicated in the material and method
169 section and they used water as liquid of conservation and not EswabTM (Gao et al. 2009). This
170 experiment then showed that recovery of bacteria whatever the species observed is more
171 efficient when aliquots are performed before freezing at -80°C. We then performed a second
172 experiment of freezing of aliquots of four other samples containing *E. coli* isolates
173 exclusively. Each aliquot was defrosted once each month during three months. We observed a
174 total recovery in this case of *E. coli* isolates. This experiment confirmed that freezing of
175 aliquots could be performed to recover *E. coli* isolates.

176 Several limits are present. First the number of samples in the two experiments are very
177 low, consequently we analyzed the viability of 3 species in the first experiment (*E. coli*, *K.*
178 *pneumoniae* and *E. faecalis*) and only one species (*E. coli*) with limited isolates in the second
179 experiment. However, these species are the main aerobic ones in the digestive tract. In
180 conclusion, this study needs to be developed with more specimens but is a real proof of
181 concept.

182 The best way to obtain quality results for both molecular and culturomic methods is to
183 use fresh stools from individuals as samples. This procedure can be easily performed when
184 the subjects of a study are healthy individuals or laboratory animals. However, fecal
185 collection when study subjects are patients may be more difficult to manage. The use of rectal
186 swabs followed by freezing seems to be the preferred procedure in view of the difficulty of

187 managing both the patient in clinical wards and the laboratory samples. In fact, adequate
188 strategies are needed to limit the biases due to changes in microbial communities during
189 sampling and storage. Rectal swabs are relatively simple samples to collect and are easily
190 transported to the laboratory (Bassis et al. 2017). They are commonly used in clinical services
191 to identify enteropathogens or to detect the presence of MDROs by culture. Knowing that
192 some of them, like ESwab™ devices, are also suitable for molecular analysis, they could be
193 used to study the composition of the fecal microbiota. In addition, studies have shown that
194 rectal swabs are a practical and acceptable alternative to stool for digestive microbiota
195 analysis (Tedjo et al. 2015; Song et al. 2016; Bassis et al. 2017).

196 In some cases, the determination of the relative density of aerobic bacteria is of
197 particular interest for specific clinical questions, whether for punctual or longitudinal
198 monitoring. For example, fecal density of ESBL-producing *E. coli* has been observed as a
199 significant risk factor for these MDROs (Ruppé et al. 2013). The method of transport and
200 conservation of fecal samples is therefore crucial before analysis as well as the culture
201 conditions (mainly the media).

202 To our knowledge, this study is the first to study the effect of F/T cycles and
203 prolonged freezing of ESwab™ liquid on the viability of aerobic bacteria. Optimal results are
204 obtained when the ESwab™ liquid is aliquoted and stored at -80 ° C. However, due to the
205 limited number of samples observed and in particular for other species than *E. coli*, our study
206 should be completed, to larger scale, by testing more samples.

207 List of figures and Table:

208 Figure 1: Boxplots of the relative quantification (in %) of aerobic bacteria from 9 ESwab™
209 samples from the first experiment evaluated for 3 weeks and processed by 2 different methods

210 A and B. Briefly, Method A consisted of treating the samples with F/T cycles for 3 weeks
211 while Method B consisted of an initial aliquoting of the samples for thawing after a single
212 freezing of each aliquot for up to 3 weeks. In the boxplot diagram, the X axis represents time
213 in weeks and Y axis the relative quantification of each bacterium (*E. faecalis*, *E. coli*, *K.*
214 *pneumoniae*) calculated by the ratio of the quantification of each bacterium at a time T
215 divided by the quantification of this bacterium at TO and multiplied by 100, then expressed
216 in percentage. When a line replaces a box plot, this means that there is no variation in the ratio
217 results for the species isolate.

218 Table1: Initial quantification of aerobic bacteria in the ESwab™ samples from both
219 experiments. The numbers correspond to the numbers of colony forming unit per mL of
220 ESwab™ liquid.

221

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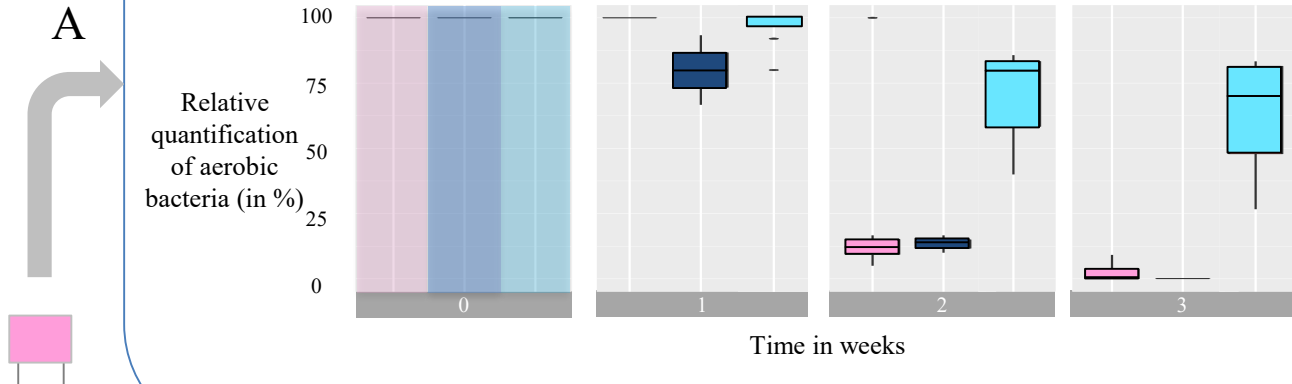
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276

277



- ESwab™ sample
- Freezing
- Thawing
- Aliquot of ESwab™ sample
- Escherichia coli*
- Klebsiella pneumoniae*
- Enterococcus faecalis*

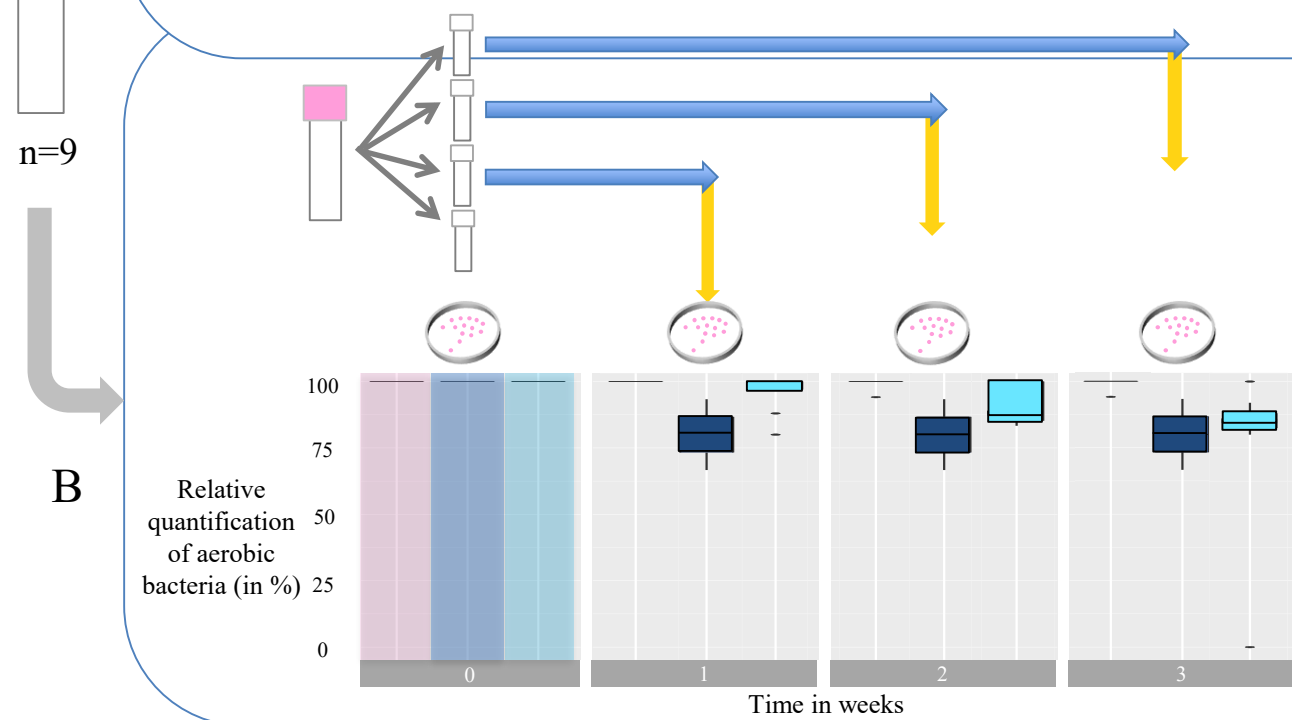


Table1: Initial quantification of aerobic bacteria in the 13 ESwab™ samples of the two experiments. The numbers correspond to the numbers of colony forming unit per mL of ESwab™ liquid.

Experiment	Sample	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Enterococcus faecalis</i>
1	1.1	1.2 10 ⁵	ND	1.1 10 ⁵
1	1.2	5.5 10 ⁶	ND	2.5 10 ⁵
1	1.3	8.5 10 ⁶	ND	1.2 10 ⁵
1	1.4	2.0 10 ⁵	ND	1.5 10 ⁷
1	1.5	ND	ND	7.0 10 ⁶
1	1.6	ND	7.5 10 ⁶	2.0 10 ⁴
1	1.7	2.0 10 ⁵	ND	2.5 10 ⁶
1	1.8	ND	3.0 10 ⁵	6.0 10 ⁶
1	1.9	8.2 10 ⁵	ND	ND
2	2.1	5.0 10 ⁶	ND	ND
2	2.2	9.0 10 ⁵	ND	ND
2	2.3	2.0 10 ⁶	ND	ND
2	2.4	1.010 ⁵	ND	ND

ND: not detected