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

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

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

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

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* Corresponding author

Abstract

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

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

Introduction:

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

Lastours et al. 2016).

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

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

Materials and Methods:

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

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

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

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

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

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

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

Results

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

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

In the second experiment, four samples each divided in three aliquots of ESwab™ fluid containing exclusively *E. coli* isolates were frozen for three months to specifically determine for this species, for a longer period, whether stability could be achieved. Each aliquot was thawed every month and the relative quantification calculated. We observed that *E. coli* viability was excellent when one F/T cycle was performed. The quantification of *E. coli* isolates in the four samples presented a mean value of $2 \cdot 10^6$ CFU / mL (ranged from $1.0 \cdot 10^5$ to $5.0 \cdot 10^6$) at TO (Table 1) and was maintained over the course of three months.

Discussion and conclusion

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

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

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

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

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

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

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

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

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

List of figures and Table:

Figure 1: Boxplots of the relative quantification (in %) of aerobic bacteria from 9 ESwabTM 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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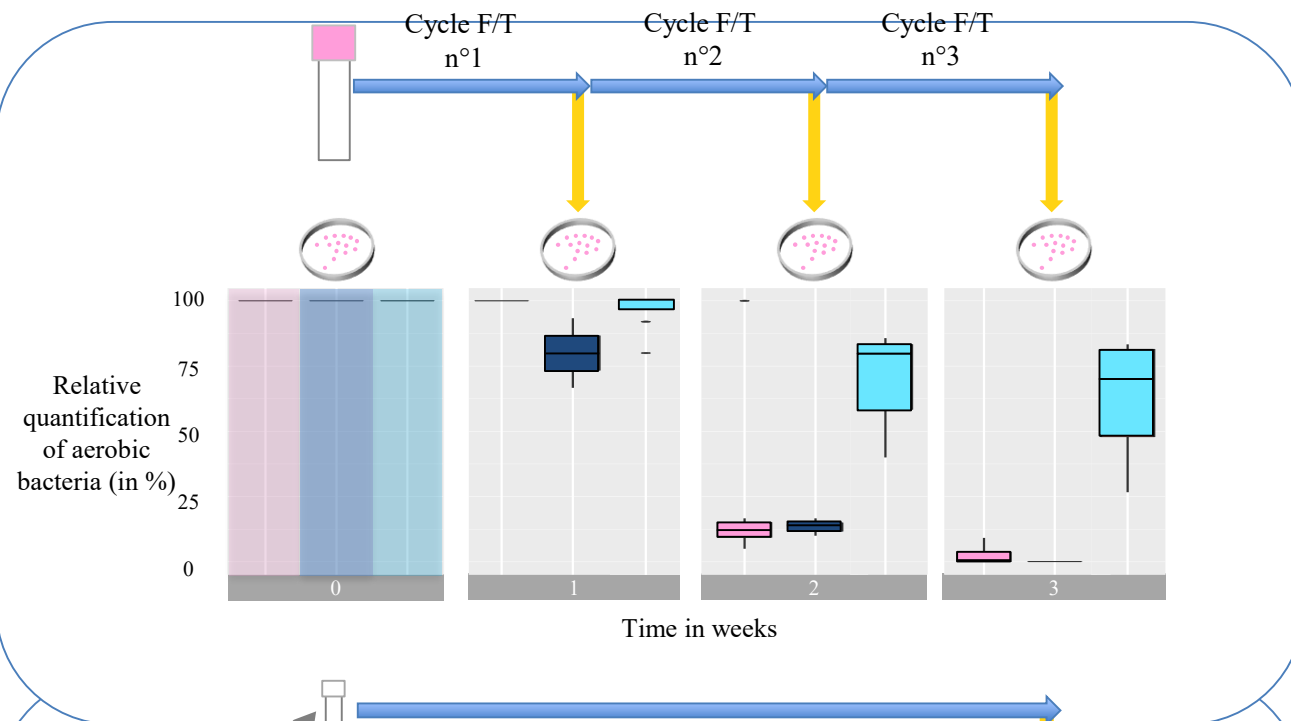
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276

277

A



n=9

B

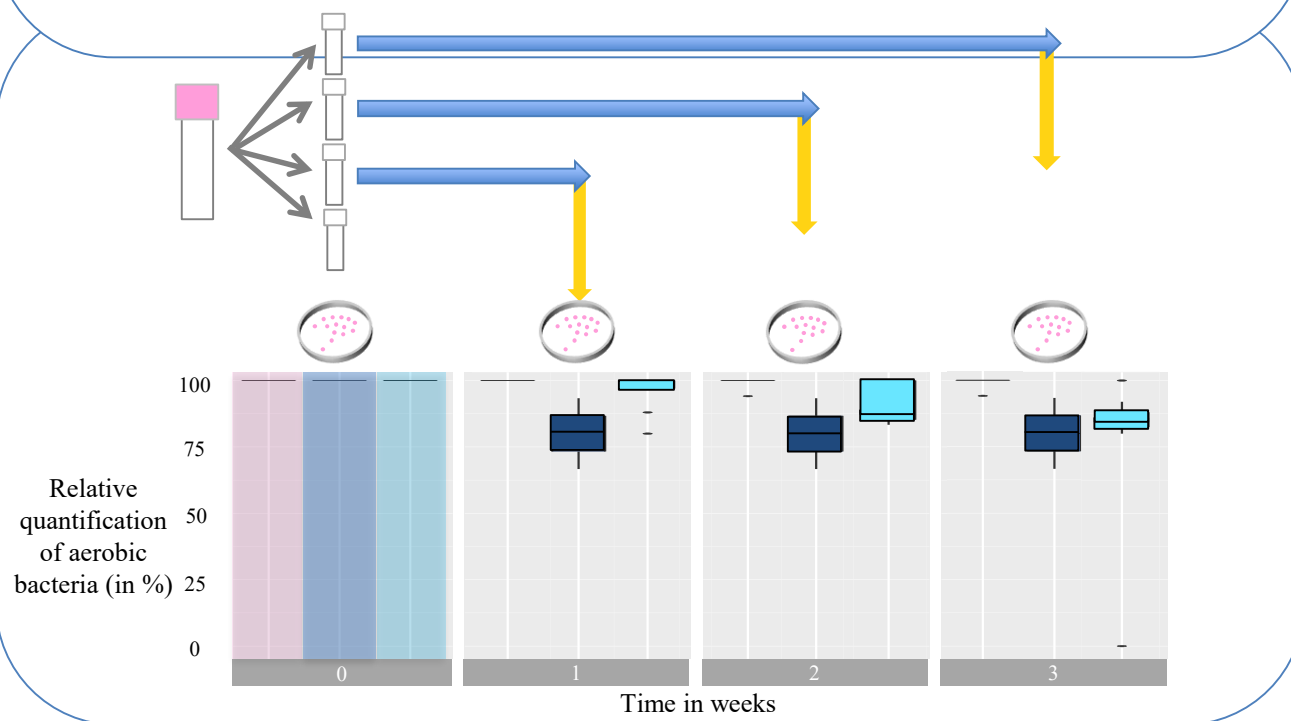


Table1: Initial quantification of aerobic bacteria in the 13 ESwab™ samples of the two experiments. The numbers correspond to the numbers of colony forming unit per mL of ESwab™ liquid.

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