academicJournals
Vol. 14(1), pp. 38-41, 7 January, 2015
DOI: 10.5897/AJB2014.13668
Article Number: BD4114149433
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

The effects of storage conditions on the viability of enteropathogenics bacteria in biobanking of human stools: Cases of *Yersinia enterocolitica*, *Salmonella enterica* Typhimurium and *Vibrio cholerae* O: 1

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Received 28 January, 2014; Accepted 20 June, 2014

Long-terms recoverability of enteropathogens is necessary for future epidemiological studies to screen stool samples when conditions do not permit immediate processing. The aim of this study was to determine the viability and the recoverability of three enteropathogens bacteria (*Yersinia enterocolitica*, *Vibrio cholerae* O: 1 and *Salmonella enterica* Typhimurium) artificially contaminated at 10^7 CFU/ml in human stool samples after our storage conditions. Preservations media used were saline, phosphate buffered saline (PBS) and Modified PBS without glycerol and containing 10 and 20% glycerol at three temperatures (4, -20 and -80°C). The viabilities and the recoverabilities of these strains were determined (weekly and monthly) respectively by plating onto tryptic soy agar and detection from artificial inoculated stools samples onto specifics agars. Bacteria populations decreased by 1-5 $\log_{10}$ CFU/ml depending on the strains tested, on the preservation media, on the glycerol concentration and the storage condition. The greatest population decrease was observed in the first week of storage at +4°C and freezing at -20°C and a slow decline in survival occurred thereafter. No organisms were recovered after one month from samples at these temperatures. When samples were subjected to storage at -80°C, all enteropathogenics bacterial were recovered after nine months storage.

Keys words: Biobanking, enteropathogenics bacteria, storage conditions, preservation media, Côte d'Ivoire.

INTRODUCTION

In developing countries, diarrheal diseases are a major public health problem that particularly affects children.

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Due to the very high infant mortality in the world more often in African countries (Boschi-Pinto et al., 2008), it is necessary to acquire knowledge on the etiological and epidemiological characteristics of diarrhea (Randremanana et al., 2012) and to have a clear view of the situation in order to design specific therapeutic measures and control strategies.

However, the ability to detect an etiologic agent is lack in many parts in remote regions of the world, especially where diarrheal disease is the most common and where most infant mortality occurred (Petti et al., 2006). Transportation of clinical specimens to specialized centers and long-term recoverability of enteropathogens are also a major problem in bacterial diagnosis, particulary when the conditions do not permit the immediate processing (Acha et al., 2005).

The long-term storage of stools samples or rectal swabs is therefore useful to carry out studies in several research field. That can also provide an opportunity to reassess later old specimens to screener emerging or remerging pathogens. Methods for the long-term storage of bacterial strains were well established, and several methods are available. Previous studies on the effect of storage conditions are laid mainly on the survival of bacteria (Yamamoto and Harris, 2001; Gorman and Adley, 2004; Ternent et al., 2004), but very few experimental studies have been conducted on the storage of biological polymicrobial specimens especially stools samples.

Freezing is the preferred method for storage of specimens of faeces but the problem with this method is the harm caused by the freezing and thawing according to several authors (Freeman and Wilcox, 2003; Acha et al., 2005).

In Côte d'Ivoire with limited financial resources, the freezing at -20°C is the most storage widely used in many health centers. Also the use of non expensive preservation media is suitable for storage of stools samples.

The aim this study was to determine a pretreatment method and storage conditions of stools samples effects on the viability of enteropathogenics bacterial strains during their biobanking.

MATERIALS AND METHODS

Sampling and bacteria strains

This study was carried out from February to November 2013 at the Department of biological ressources (Biobank) of the Institut Pasteur in Côte d'Ivoire. It consisted of four human stools freshly issued and three reference strains of *Yersinia enterocolitica* IP383, *Vibrio cholerae* O:1 *Salmonella enterica* Typhimurium from bio-collection of the Institut Pasteur in Côte d'Ivoire and the Pasteur Institute in Paris.

The fecal samples included in this study were free for these enteric bacteria and were collected from healthy people.

Pretreatment of fecal samples

Thiel and Blaut (2005) described the pretreatment method with some modifications. Briefly, 2 g of fresh stools were transferred into a sterile falcon tube (15 ml) and diluted in 10 ml of different preservation media which was to be tested. These preservation media were: Phosphate Buffered Saline (containing 150 mM NaCl, 10 mM NaH_2PO_4, 20 mM NaH_2PO_4, pH 7.4), the saline (0.9% NaCl), and modified PBS (0.1% peptone, 0.25% yeast extract).

These preservation media were prepared without glycerol or with glycerol at 10 and 20% in final concentration. The samples were homogenized by adding ten sterile glass beads (3 mm of diameter) and stirring for 3 min using vortex mixer (Sterilin ® STUART LTD). Thereafter, the fecal samples were centrifuged at 3000 rpm for 5 min to remove the glass beads and larger solid particles (debris) contained in the stool.

Quantification of bacterial

From a pure culture of reference strains of 18 to 24 h onto non-selective agar, a bacterial suspension was prepared in 5 ml of suspension medium (Biomerieux ®) at 0.5 Mac-Farhland equivalent to 10^8 CFU/ml using a DENSIMAT (VITEK, Biomerieux ®). The fecal samples were pretreated and the control media were contaminated with bacteria suspensions in a 1: 10 proportion (1 ml of bacterial suspension + 9 ml of stool). This gave an initial concentration of 10^7 CFU/ml in the samples. Several aliquots (1 ml) were then made and packaged in cryovials and Eppendorf tubes. Each stool sample and his control were stored at different storage temperatures (+4, -20 and -80°C) for 9 months.

Viability tests

The viability test was consisted of a simultaneous detection and counting of bacteria, respectively, in contaminated storage media and stool samples.

Before any weekly and monthly analysis, all samples were placed at room temperature for thawing. The numbering was performed after a serial of decimal dilution, and 0.1 ml was spread onto tryptic soy agar TSA (Biorad). For bacterial detection a loopful of test contaminated stool samples was streaked on culture medium appropriate to the strain to be recovered:TCBS agar (Scharlau) for *Vibrio cholerae*, Hecktoen agar (Lyofilchem) for *Salmonella enterica* and CIN agar (Cefsulodin Irgasan Novobiocin, *Yersinia* Selective Agar Base and *Yersinia* Supplement, Liofilchem) for *Yersinia enterocolitica*.

All agar plates were incubated at 37°C except CIN agar which was incubated at 30°C. The number of colony forming unit (CFU) of each preservation medium was determined by the following formula according the French Nor (AFNOR, 2002):

$$N\left(CFU/ml\right)=\frac{\sum C}{V\left(n_1+0,1n_2\right)d}$$

Where, ΣC is the The total of all colonies counted on the agar plate in two serial decimal dilutions; V is the volume of the inoculum applied on agar plate; n1 is the the number of plate retained for the first considered dilution; n2 is the the number of plate retained for the second considered dilution and d is the the first dilution considered.

Statistical analysis

To compare the results of all assays, statistical analysis was carried

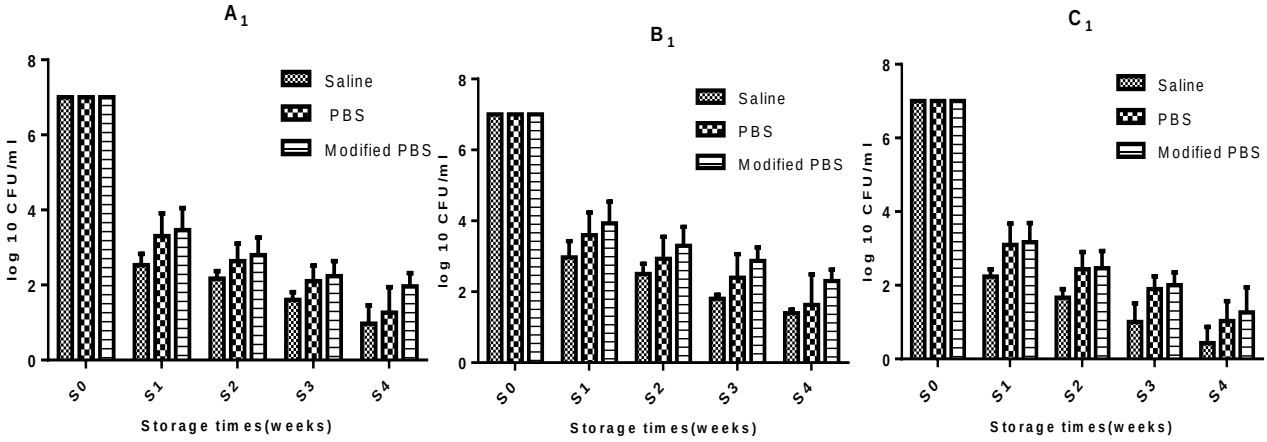

Figure 1. Recovery of enteropathogenics bacteria stored in continuous cyclic frozen without thawing in the preservation media at -20°C. **A_1**,*Yersinia enterocolitica* IP383. **B_1,** *Salmonella enterica* Typhimitium. **C_1**:*Vibrio cholera* O:1.

out using a GraphPad Prism version 5 software. The values reported define the boundaries of an interval with 95% certainty. The results were only considered statistically different at $P < 0.05$.

RESULTS AND DISCUSSION

To date, there is no ideal medium that preserves the viability of pathogens and flora without allowing the growth or inhibition of some species (Wasfy et al., 1995). The preservation of pathogen viability in biological specimens is also essential for their recovery. This is especially true for faecal specimens; overgrowth of normal faecal flora can mask enteropathogenics and impair isolation.

In our study, after storage at 4°C, most strains were not viable for more than one week. Exceptionally *Yersinia enterocolitica* was viable and recoverable in all preservation media more than one month. These results are comparable to those of Dan et al. (1989) when a transport medium was not used. The ability of *Y. enterocolitica* to grow at low temperature due to its psychrotrophic character but the other bacteria strains are mesophilic.

In our study, the recoverability and viabibity of enteropathogenics species at -20°C were ambiguous. The directly frozen of stools samples without cryoprotective agent (glycerol) in the preservation media at -20°C was catastrophic for all enteropathogenics bacteria.

Poor results were obtained when stools samples were frozen for one week at -20°C. With the greatest glycerol concentration (20%) *Vibrio cholerae*, *Y. enterocolitica* and *Salmonella enterica* Typhimirium underwent globally 3 to 4,7 $\log_{10}$ CFU/ml population decline after one week storage in the different preservation media (Figure 1). Non-significant result was obtained between all the preservation media. The different bacteria species may vary in their susceptibility to damage by freezing and thawing.

At this low temperature, the pH and biochemical activities of stool microbiota (aerotolerant anaerobes) have been reported to be detrimental to the viabilities of pathogenic organisms in fecal samples (Lennette et al., 1985).

All the enteropathogenics species tested were preserved at -80°C for the entire period of study and quantitative recovery was very satisfactory whereas there was a difference between the results obtained with the various preservation media, and between storage with the different glycerol concentration in the preservation media. All preservation media with 20% glycerol gave also better results.

The loss of viability was also strain-dependent (p<0.05). In this study, *Salmonella enterica* was better recovered than *Y. enterocolitica* followed by *Vibrio cholerae. V. cholerae* was the most sensitive strains exhibiting 3,8 to 4,9 $\log_{10}$ CFU/ml decrease comparatively to *Yersinia* and *Salmonella* strains whose showed respectively 2,9 to 4 $\log_{10}$ CFU/ml and 2,3 to 2.9 $\log_{10}$ CFU/ml decrease during the study period.

The comparison of bacterial strains sustainability, viability and forever recoverabilty during this storage method in preservation media showed that there was not a difference between saline and PBS and between PBS and modified PBS for all bacterial strains. The difference was observed only between saline and modified PBS. A significant difference ($p<0.05$) was observed at the seventh month storage for *Y. enterocolitica* strain and this difference was extremely significant ($P<0.01$) during the two last months (Figure 2). Right from the fourth month storage, difference was observed for Salmonella and after six (6) months storage, the decrease was similar in the all preservation media. That also has been observed

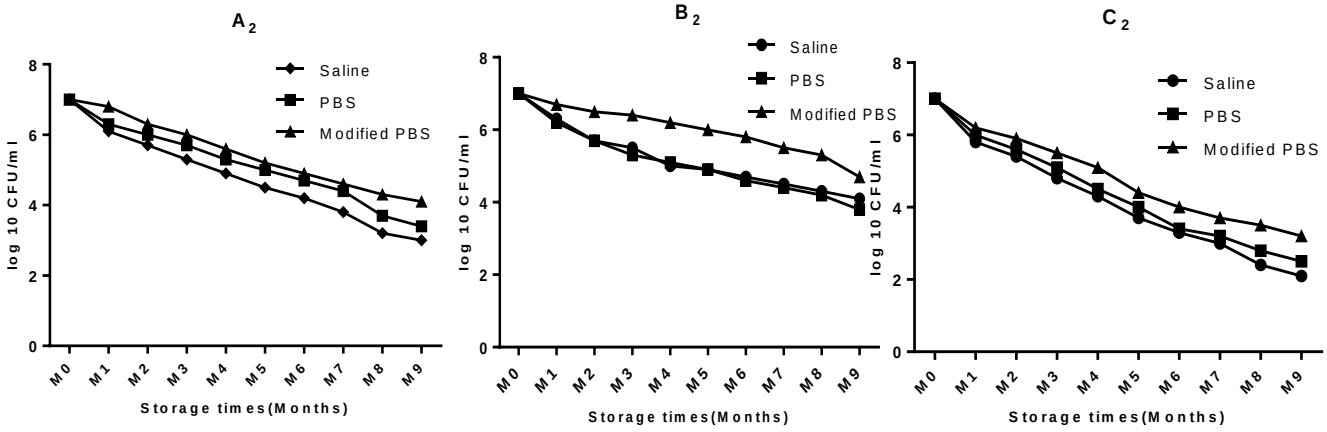

Figure 2. Enteropathogenics bacteria recovered in continuous cyclic frozen in the preservation media with 20% glycerol at -80°C. **A_2,** *Yersinia enterocolitica* IP383. **B_2.** *Salmonella enterica* Typhimitium. **C_2,** *Vibrio cholera* O:1.

for *V. cholerea* strain but from the eighth month storage this decrease was more significant.

This observation is valid for all bacteria studied but strains *of V. cholerae* seems to lose viability quickly, and the long-term cultivability. Conversely, when stool samples were stored at -80°C in buffered media the viability and recoverability of enteropathogenics bacteria were better compared to those obtained without storage medium and physiological saline. The sharpest decline in viability was observed in saline and PBS but better recoverability seems to be obtaining with modified PBS media. This suggestion may disagree with the view that the low nutrient content of Carry Blair and utilization of phosphate as a buffering agent may provide suitable sample viability and control of contaminants that may mask the recovery of true pathogens.

We conclude that most enteropathogens can survive in faecal samples for as long as nine months when stored only at lowest temperature -80°C. These bacteria can also be preserved, at least partially, for the same period, if preservation media are used in conjunction with low temperatures.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

We thank Dr. Elisabeth CARNIEL (Centre National des *Yersinia*, Institut Pasteur, Paris, France) for the reference strains.

REFERENCES

Acha SJI, Kühn G, Mbazima P, Colque-Navarro RM (2005). Changes of viability and composition of the *Escherichia coli* flora in faecal samples during long time storage. J. Microbiol. Methods 63:229-238

AFNOR (2002). Microbiologie alimentaire, 8e édition. Saint-Denis LaPlaine Cedex. Tome 1:676p.

Boschi-Pinto C, Velebit L, Shibuya K (2008) Estimating child mortality due to diarrhoea in developing countries. Bull World Health Organ 86:710-717.

Dan M, Richardson J, Miliotis MD, Koornhof JH (1989). Comparison of preservation media and freezing conditions for storage of specimens of faeces. J. Med. Microbiol. 28:151-154.

Freeman J, Wilcox MH (2003). The effects of storage conditions on viability of *Clostridium difficile* vegetative cells and spores and toxin activity in human faeces. J. Clin. Pathol. 56(2):126-128.

Gorman R, Adley CC (2004). An evaluation of five preservation techniques and conventional freezing temperatures of -20 and -85°C for long-term preservation of *Campylobacter jejuni.* Lett. Appl. Microbiol. 38:306-310.

Lennette EH, Balows A, Hausler WJ, Shadomy HJ (1985) **.**Manual of clinical microbiology, 4th edn. American Society for Microbiology , Washington D. C.

Petti CA, Polage CR, Quinn TC, Ronald AR, Sande MA (2006). Laboratory medicine in Africa: a barrier to effective health care. Clin. Infect. Dis. 42:377-382. doi: 10.1086/499363.

Randremanana R, Randrianirina F, Gousseff M, Dubois N, Razafindratsimandresy R, et al. (2012) Case-Control Study of the Etiology of Infant Diarrheal Disease in 14 Districts in Madagascar. PLoS ONE 7(9):e44533. doi:10.1371/journal.pone.0044533

Ternent HE, Innocent GT, Filshie LM, Taylor DJ, Steele WB, McEwen SA, Reilly GJ, Reid SW, Mellor DJ (2004). Frozen storage of *Escherichia coli O157* in buffered peptone water and its detection on bovine carcasses. J. Food Prot. 67 (1):40- 45.

Thiel R, Blaut M (2005). An improved method for the automated enumeration of fluorescenty labeled bacteria in human feaces. J. Microbiol. Methods. 61:369-379

Wasfy M, Buhari O, Atef E, Albert C (1995). Comparison of Preservation Media for Storage of Stool Samples J. Clin. Microbial. 33:2176-2178

Yamamoto SA, Harris LJ (2001). The effects of freezing and thawing on the survival of *Escherichia coli O157:H7* in apple juice. Int. J. Food Microbiol. 67(2):89-96.