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Optimization of Technical Parameters for Detecting Mycobacteria in Hospital Wastewater in Tropical Urban Areas: The Case of the City of Abidjan (Côte d'Ivoire)

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Abstract

The loads of organic matter, microorganisms, detergents and antibiotics in liquid hospital effluents make them complex environments, raising numerous health and ecological questions. Investigations of mycobacteria in water lack adequate techniques. This study is the first part of a pilot project aimed at developing an optimized protocol for the isolation of mycobacteria from hospital effluents, as a prelude to more in-depth investigation in this matrix. The aim was to compare the performance of two decontamination methods, three culture media and two incubation temperatures generally proposed in the literature, in order to identify the most effective methods in each case, as well as possible areas for improvement in the isolation of these germs from this environmental matrix. The results show that liquid hospital effluent can be decontaminated using both the NaOH method (4%; for 30 min.) and the CPC method (0.05%; for 30 min.), with the same mycobacteria recovery efficiency. Despite the low concentration, decontamination with CPC killed more mycobacteria and sufficiently eliminated contaminating germs. In contrast, decontamination with NaOH was less harmful to mycobacteria, but did not remove many contaminating germs. On the other hand, LJG medium per-

formed better than LJGF medium and LJGP medium for the growth of mycobacteria in hospital waters. Finally, there was no difference in performance between the two incubation temperatures of 30˚C and 37˚C. The results of this study show that further evaluation of existing protocols is required in order to optimize methods for the pre-treatment of hospital effluent for the isolation of mycobacteria.

Keywords

Hospital Wastewater, Mycobacterium, Decontamination, Löwenstein Jensen Medium, Growth Temperature

1. Introduction

In recent years, non-tuberculous mycobacteria (NTM) infections have increased considerably, constituting a public health problem of varying degrees in different parts of the world [1] [2].

The reasons for the proliferation of these opportunistic pathogens in the environment remain to be understood. Among environmental fluids, water is particularly cited as a major source of mycobacteria [2] [3] [4].

In hospitals, NTMs are found at various levels, including water distribution networks, certain medical devices and patient excreta [5] [6]. Within hospital effluents, mycobacteria are likely to persist due to three factors described in the literature: their resistance to chlorine disinfection, their colonization of biofilms and their association with certain free-living amoebae [7] [8] [9]. While NTMs are opportunistic pathogens causing nosocomial infections, few studies to date have focused on assessing the performance of existing protocols for isolating these germs from hospital wastewater [10] [11] [12].

In water, the lack of suitable techniques for isolating mycobacteria has largely limited investigations into these bacteria. The problem can be even more complex with hospital effluents, due to the various inorganic substances they contain. The nature of the chemical relationships between these substances and decontamination products, on the one hand, and the components of culture media, on the other, is not always known. Combinations of certain chemical substances can be harmful when they interact [13].

In Côte d'Ivoire, mycobacterial infections are a real public health problem [14] [15] [16]. As part of a program to study pathogens in water, work was initiated on mycobacteria, and a study was carried out to develop a protocol for isolating these germs from hospital effluents. The present work reports the results of one of the components of this study. The objective was to evaluate the performance of two decontamination methods, three growth media and two incubation temperatures for the isolation of mycobacteria from hospital wastewater.

These different methods have been selected for this study because they are generally proposed in the literature and are easy to implement.

2. Materials and Methods

2.1. Collection of Hospital Effluent Samples

60 samples of liquid hospital effluent collected between January and December 2020 from 4 hospitals in Abidjan were analyzed for mycobacteria. Samples were collected from the main manholes (collecting effluent from all other hospital manholes) once a week, then transported within 3 hours at +4˚C to the laboratory for immediate culture.

2.2. Isolation of Mycobacteria

Samples were analyzed in parallel by twelve methods for mycobacterial isolation. These twelve methods were a particular combination of two decontamination methods, three growth media and two incubation temperatures for mycobacterial isolation (Table 1).

2.2.1. Decontamination Methods

Two different decontamination methods were used in this study: the Cetylpiridium Chloride method (CPC; 0.05%) and the Petroff method (NaOH; 4%). Samples were concentrated as previously before decontamination [17]. For the CPC method, 10 ml of the previously concentrated sample was brought into

Table 1. Methods used to isolate mycobacteria.

Method	Technical parameters			
	Decontamination	Growth medium	Incubation temperature	Number of samples
M1	CPC (0.005%)[a]	LJG[c]	30˚C	60
M2	CPC (0.005%)[a]	LJGF[d]	30˚C	60
M3	CPC (0.005%)[a]	LJGP[e]	30˚C	60
M4	CPC (0.005%)[a]	LJG[c]	37˚C	60
M5	CPC (0.005%)[a]	LJGF[d]	37˚C	60
M6	CPC (0.005%)[a]	LJGP[e]	37˚C	60
M7	NaOH (4%)[b]	LJG[c]	30˚C	60
M8	NaOH (4%)[b]	LJGF[d]	30˚C	60
M9	NaOH (4%)[b]	LJGP[e]	30˚C	60
M10	NaOH (4%)[b]	LJG[c]	37˚C	60
M11	NaOH (4%)[b]	LJGF[d]	37˚C	60
M12	NaOH (4%)[b]	LJGP[e]	37˚C	60

[a]Cetylpiridium Chloridewith a final volume of 0.005%; [b]Sodium hydroxide with a final volume of 4%; [c]Glycerol-containing LJ medium; [d]Glycerol + Iron-containing LJ medium; [e]Glycerol + pyruvate-containing LJ medium.

contact with 10 ml of CPC (0.1%) to obtain a final concentration of 0.05%. The mixture was gently shaken for 30s to homogenize. After 30 min of contact, the suspension was neutralized by adding 10 mL of sterile distilled water before being centrifuged at 3000 rpm for 15 min (4˚C). The resulting pellet was resuspended in 2 mL sterile distilled water, then 200 µl were inoculated with culture media for mycobacterial isolation. For the Petoff method, 10 ml of the previously concentrated sample was brought into contact with 10 ml NaOH (4%). The mixture was stirred using a Kahn shaker. After 30 min of contact, the suspension was neutralized by adding 10 mL of neutralizing solution before centrifugation at 3000 rpm for 15 min, 4˚C. The resulting pellet was resuspended in 2 ml sterile distilled water, then 200 µl were inoculated with culture media for mycobacterial isolation.

2.2.2. Growth Media

For primary culture, three variants of Löwenstein Jensen medium were used: LJG, LJGF (glycerol, with 1 to 2.5% w/v ammoniacal iron (III) citrate) and LJGP (glycerol, with 0.4% sodium pyruvate). Growth media were prepared as inclined media by the laboratory input preparation unit of the institut pasteur de Côte d'Ivoire from Löwenstein Jensen powder (BIO-RAD, France, ref. 69675), ammoniacal iron (III) citrate powder (SIGMA-ALDRICH, France, ref: F5879) sodium pyruvate powder (SIGMA-ALDRICH, France, ref.: P5280) according to the manufacturer's recommendations. After preparation, the media also underwent quality control tests as recommended by the manufacturer.

2.2.3. Culture Conditions and Identification of Mycobacteria

Media were incubated at 30˚C and 37˚C for 12 weeks, ensuring that the screw caps on the culture tubes were loose enough to allow a certain degree of gas exchange, and tight enough to prevent visually observable desiccation of the media. Cultures were examined daily for the first week and weekly for the remainder of the time for microbial growth. When a colony was detected, it was tested for resistance to acid and alcohol by Ziehl-Neelsen staining. In the event of positive staining, the strain was confirmed as a mycobacterium by PCR sequencing of the 16S - 23S RNA gene region, as above, using a pure subculture with sufficient growth [18]. When the colony was identified as a mycobacterium, its isolation medium was then counted as a positive medium. A culture was flagged as contaminated and. A culture was classified as negative if no growth was detected in the cultures after 12 weeks.

2.2.4. Data Analysis

From the total number of samples tested per method, the positivity, negativity and contamination rates for each isolation method were determined, representing up to 100% for each method. For a given method, the positivity rate corresponded to the proportion of samples producing mycobacterial colonies, the negativity rate indicated the proportion of samples producing no microbial growth, and the contamination rate represented the proportion of samples from which

no mycobacteria could be isolated during primary culture due to non-mycobacterial proliferation (contamination) of the medium or due to liquefaction of the medium.

Once the results had been collected, the data from the two decontamination methods, the data from the three culture media and the data from the two incubation temperatures were compared with each other to determine the best decontamination method, the best growth medium and the best incubation temperature for isolating mycobacteria.

2.2.5. Statistical Method

In addition to the descriptive statistics for the parameters number of contaminations, number of negatives and number of positives for each of the decontamination methods, culture medium and incubation temperature in our study, comparison tests of proportions based on the Khi-Deux statistic (χ^2) were carried out using R software. With this software, comparisons between two proportions and comparisons of more than two proportions were carried out using the prop.test and pairwise.prop.test functions respectively. At the 5% threshold, these tests lead to significant alternative hypotheses when the p-value is less than 0.05.

3. Results

3.1. Distribution of Results According to Methods Evaluated

A total of 360 hospital effluent samples were decontaminated in parallel by the CPC and NaOH methods, 240 effluent samples were cultured in parallel on LJG medium, LJGF medium and LJGP medium, and 360 hospital effluent samples were incubated in parallel at 30˚C and 37˚C. The performance of each decontamination method, culture medium and incubation temperature was presented (**Table 2**).

3.2. Results of the Decontamination Method Performance Comparison

A comparison of the performance of the decontamination methods shows that there is no significant difference between the positivity rate of the CPC method and that of the NaOH method ($p > 0.05$). However, the contamination rate of the CPC method (41.7%) is lower than that of the NaOH method (58.3%) ($p < 0.05$). Similarly, the negativity rate of the CPC method (35.8%) was higher than that of the NaOH method (16.7%) ($p < 0.05$) (**Table 3**).

3.3. Results of Growth Media Performance Comparison

A two-by-two comparison of the performance of LJG and LJGF medium shows that these two culture media have virtually identical positivity and contamination rates ($p > 0.05$). Nevertheless, the negativity rate of LJG medium remains lower than that of LJGF medium ($p < 0.05$).

A two-by-two comparison of the performance of LJG and LJGP media shows that the positivity and contamination rates of LJG media are better than those of LJGP media. However, the negativity rate of LJG medium is higher than that of LJGP medium ($p < 0.05$) (Table 4).

3.4. Results of Incubation Temperature Comparison

A comparison of the performance of the 30˚C incubation method and that of the 37˚C incubation method shows that there is no significant difference between positivity rates, contamination rates and negativity rates (Table 5).

Table 2. Breakdown of results by method evaluated.

Evaluated method	N	Positivity	Contamination	Negativity
CPC decontamination	360	81 (22.5%)	150 (41.7%)	129 (35.8%)
NaOH decontamination	360	90 (25.0%)	210 (58.3%)	60 (16.7%)
Culture on LJG medium	240	75 (31.3%)	100 (41.7%)	65 (27.1%)
Culture on LJGF medium	240	58 (24.2%)	89 (37.1%)	93 (38.8%)
Culture on LJGP medium	360	38 (10.6%)	171 (47.5%)	31 (8.6%)
Incubation at 30˚C	360	74 (20.6%)	186 (51.7%)	100 (27.8%)
Incubation at 37˚C	360	97 (26.9%)	174 (48.3%)	89 (24.7%)

Table 3. Performance comparison of decontamination methods.

	EVALUATION (%)		p-value
	CPC (0.005%)	NaOH (4%)	
Positivity	22.5	25	>0.05
Contamination	41.7	58.3	<0.05
Negativity	35.8	16.7	<0.05

Table 4. Comparison of growth media performance.

	EVALUATION (%)			P1[a]	P2[b]	P3[c]
	LJG	LJGF	LJGP			
Positivity	31.3	24.2	15.9	<0.05	>0.05	<0.05
Contamination	27.1	38.8	12.9	<0.05	<0.05	<0.05
Negativity	41.7	37.1	71.3	<0.05	>0.05	<0.05

[a]P value calculated to compare the three growth media LJG, LJGF and LJGP; [b]P value calculated to compare LJG and LJGF; [c]P value calculated to compare LJG and LJGP.

Table 5. Performance comparison of incubation methods.

	EVALUATION (%)		p-value
	30˚C	37˚C	
Positivity	20.6	26.9	>0.05
Contamination	27.8	24.7	>0.05
Negativity	51.7	48.3	>0.05

4. Discussion

In order to determine the best decontamination method, growth medium and incubation temperature for isolating mycobacteria from hospital effluents, 60 wastewater samples from 4 hospitals in Abidjan were analyzed in parallel using two decontamination methods, three growth media and two incubation temperatures. The results of the different methods were compared.

4.1. Analysis of Decontamination Method Data

Sample decontamination is an important step prior to culture, to eliminate contaminating germs while preserving the mycobacteria. Because of their more rapid growth, some of the microorganisms in effluent pose a major problem to the detection and enumeration of slower-growing mycobacteria by culture [19]. Some decontamination agents destroy significant numbers of mycobacteria along with the contaminants, while others are too weak to destroy them [20]. The identification of optimal decontamination methods for the isolation of NTMs from hospital effluents is essential to improve prevention and control practices [21].

CPC and NaOH are decontamination agents commonly found in the literature for the pretreatment of water samples prior to mycobacterial culture. CPC is recommended by the European Centre for Disease Prevention and Control for decontamination of water samples, while the standard method for decontamination of sputum samples uses NaOH [22]. Several previous studies have demonstrated that treatment of drinking water and surface water samples with 0.004 or 0.005% CPC has a higher mycobacteria recovery efficiency and a lower contamination rate than using 1% - 3% NaOH [23].

With respective positivity rates of 22.5% and 25% for CPC and NaOH, the data from the present study suggest, on the contrary, that in hospital wastewater, these two decontamination methods perform equally well in isolating mycobacteria ($p > 0.005$) (**Table 3**). Similarly, as with surface water, CPC applied to hospital effluent sufficiently eliminated contaminating germs, but was more harmful to mycobacteria despite a low concentration, which was the opposite of NaOH (**Table 3**). This result may be linked to the ecotoxic and biological composition of hospital effluent. In fact, according to some studies, the performance of CPC and NaOH in recovering mycobacteria from certain matrices varies according to

the predominant mycobacterial species and the organic matter content, respectively [19] [20]. In addition, it has been shown that the effectiveness of different decontamination methods can vary according to the concentration of decontaminating agents used and contact times with the treated sample [12]-[23]. Some authors also report a variation in the effectiveness of decontamination methods depending on the origin of the samples, as mycobacterial species do not have the same resistance to different decontamination procedures [10]. Further studies comparing various concentrations and/or contact times of CPC and NaOH are needed to define the decontamination protocols best suited to the pre-treatment of hospital effluents.

4.2. Analysis of Growth Media

Despite the development of rapid molecular tests, culture still holds an important place among the biological diagnostic methods recommended by the World Health Organization [24] [25]. It remains the most sensitive method, paving the way for species identification and antibiotic susceptibility testing based on strain isolation [26]. Lowenstein-Jensen (LJ) medium is a solid, selective egg-based reference medium specially used for the cultivation and isolation of Mycobacterium species. This medium contains various constituents, including glycerol, which contributes to nutrient supply, with the exception of certain Mycobacteria (notably *M. bovis*), on which it exerts an inhibitory effect. In this case, glycerol is replaced by sodium pyruvate to enable the growth of inhibited strains. Other mycobacteria such as *M. haemophilum*, on the other hand, require the addition of supplementary supplements such as ammoniacal iron (III) citrate, for improved growth [27]. The various LJ variants proposed in the literature and the cultivation requirements of certain mycobacteria prompted our interest in comparing the performance of LJGF medium and LJGP medium with that of LJG medium for the isolation of mycobacteria from hospital effluents. Analysis of the data from this study shows that LJG medium is more effective for isolating mycobacteria from hospital wastewater (**Table 4**). The performance of LJG medium over other media for the cultivation of mycobacteria has also been reported by other authors [28]. It seems that the mycobacterial species present in hospital effluents in Abidjan have no particular requirements for iron, as the presence of this supplement did not improve the performance of the LJGF medium. On the other hand, the lower positivity rate and much higher contamination rate of the LJGP medium show that the addition of pyruvate would limit the growth of certain mycobacteria present in hospital effluents, probably by potentiating the effect of an inhibitor or contaminants. In their investigations on clinical samples, some authors have reached the same conclusion concerning LJGP and LJG media [29]. However, these authors found that mycobacterial growth was faster on LJGP medium (1.6 weeks) than on LJG medium (2 weeks). Although these aspects were not evaluated in the present study, the prospect of a significant reduction in labor time and input costs with LJGP medium could be promising for

the isolation of *M. tuberculosis* complex in resource-limited countries [29]. Other studies have proposed supplementing LJG medium with certain antibiotics to increase its selectivity against contaminating germs. This process would enhance the performance of LJG medium by reducing the incidence of combination [25] [26] [27].

4.3. Analysis of Growth Temperatures

Temperature is an important parameter influencing both multiplication and bacterial metabolism, by affecting the speed of biochemical reactions [30]. Some mycobacteria, such as *M. hassiacum* sp. nov., grow at 65˚C [31]. Usually, the incubation temperatures used for the detection of the majority of NTMs in the environment are 30˚C and 37˚C. These methods, adopted from clinical microbiology, deserve to be further evaluated and adapted to environmental samples [32]. In this study, a comparison of culture incubation temperatures shows that mycobacteria from hospital effluents are able to grow at both 30˚C and 37˚C. According to the literature, with the exception of certain species such as *M. ulcerans*, *M. marinum* and *M. haemophilum*, which grow at 28˚C to 30˚C, several species of mycobacteria grow at 37˚C [33]. In their various studies, Ulmann *et al.* also showed that mycobacterial growth was most optimal at 37˚C, while Neumann *et al.* reported that a higher proportion of contamination was observed on media cultured at 30˚C for the detection of mycobacteria [34] [35]. The data from this study suggest that mycobacterial species circulating in hospital effluents are capable of adapting to either 30˚C or 37˚C, depending on the case. In fact, according to ecological studies, mycobacteria are capable of maintaining and proliferating in a variety of pH and temperature conditions, while showing maximum tolerance to physico-chemical and microbiological variations in the environment [36].

5. Conclusions

The results of the present pilot study indicate that liquid hospital effluent can be decontaminated by both the NaOH method (4%; Pdt 30 mm) and the CPC method (0.05%; Pendant 30 mm), for the same mycobacteria recovery efficiency. However, despite the low concentration, decontamination with CPC killed more mycobacteria and sufficiently eliminated contaminating germs. In contrast, decontamination with NaOH was less harmful to mycobacteria, but did not eliminate many contaminating germs. Furthermore, LJG medium performed better than LJGF medium and LJGP medium for the growth of mycobacteria in hospital water. Finally, mycobacteria isolation rates did not vary according to the incubation temperatures usually used in the literature, *i.e.* 30˚C and 37˚C.

The complexity of hospital effluents loaded with various substances and pathogens could partly explain the rates of contamination or false-negative cultures that affect the performance of these methods. These initial results will contribute to the development of optimized protocols for more extensive investiga-

tions of mycobacteria in this environmental matrix.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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