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RESEARCH ARTICLE

PERFORMANCE OF THE SODIUM ACETATE METHOD IN THE EXTRACTION OF MYCOBACTERIUM ULCERANS DNA

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ABSTRACT

Background: *Mycobacterium ulcerans* infection often leads to extensive destruction of the skin and soft tissues, with extensive ulcerations usually occurring on the limbs. Confirmation of cases using gene amplification (PCR) or direct examination of smears has become an essential aspect in the overall management of the disease. **Objective:** To evaluate the sodium acetate method in the extraction of *Mycobacterium ulcerans* DNA for detection at PCR to confirm cases. Methods: Samples were taken from patients suspected of Buruli ulcer during the study period between January and April 2018 in selected sites. Analyzes were performed at the Mycobacteria laboratory using the Ziehl test, PCR by sodium acetate method and that using the Maxwell Kit. The results of the first two tests were compared to those of PCR by the Maxwell method used as a gold standard. The significance level was set at 5% and the 95% confidence interval (CI). **Results:** The proportion of positive samples was 16.5% for Ziehl, 21.7% for sodium acetate PCR and 35% for that using the Maxwell kit. PCR by the sodium acetate method yields sensitivity, specificity and VPP respectively of 52.9%, 95.2%, 85.7%. In contrast, the sensitivity, specificity and VPP for the Ziehl technique were 47.1%, 100% and 100%. **Conclusion:** Although the sensitivity is low, sodium acetate method can be used in absence of Maxwell Kit for extraction of *Mycobacterium ulcerans* DNA for detection and for confirmation of cases. It is the same for the Ziehl being a quick and simple test to perform in the case of detection.

Key words: Performance; Sodium acetate; DNA extraction; *Mycobacterium ulcerans;* Kinshasa.

INTRODUCTION

Mycobacterium ulcerans is the infectious agent responsible for the skin infection called Buruli ulcer (BU), which is both a mysterious disease and a major public health concern. The disease is chronic debilitating mainly affecting the skin and sometimes also bones. It affects mainly children under fifteen years without sex predilection. The lesions frequently affect the limbs: 35% the upper limbs, 55% the lower limbs and 10% other parts of the body (Aubry *et al*. 2017; Portaels *et al*.2014; Who 2017; Kibadi *et al*. 2014). Worldwide, the disease is found in 33 countries in Africa, Americas and Western Pacific, mainly in the tropics, according to partial data from 13 countries, there were 2206 cases in 2017 and 1920 in 2016 (Aubry *et al*.2017; Janssens *et al*.2005; Who 2017). More cases are in Australia and Nigeria, and it should be noted that the majority of cases in Africa are reported in some West and Central African countries notably in Benin, Cameroon, Ivory Coast, Ghana and the Democratic Republic of Congo (DRC) (Aubry *et al*.2017). DRC has 17 endemic health zones (3%), 26 are suspected to be endemic (5%) and 472 are non-endemic (92%) according to the 2014 mapping (Kibadi *et al*. 2014). This mapping shows that 7 provinces in the DRC are affected by the Buruli ulcer and most cases are reported in Kongo Central province (Kibadi *et al*. 2014). The World Health Organization (WHO) reports that the number of cases of Buruli ulcer detected and reported in DRC from 2002 to 2013 was 2,363 (Kabadi *et al*. 2014).

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Thus, early diagnosis and treatment are the only means of reducing morbidity and avoiding long-term disability (Aubry *et al*. 2017). Among the tests recommended by the WHO for case confirmation, there is the gene amplification (PCR) of the IS2404 sequence which allows the results to be obtained early and rapidly in order to ensure a better management of the patients by reducing morbidity and disability (Aubry *et al*. 2017; Bretzel *et al*. 2007). Several methods are used for the extraction of *Mycobacterium ulcerans* DNA which will be amplified and hybridized thereafter to allow its detection. Among these methods, Maxwell is a reference for its realization semi-automated equipment requiring financial means for its acquisition. This could be sometimes an obstacle to its systematic use in resource-limited countries for lack of adequate budget. However, this difficulty can be overcome by using other simple manual methods of extracting existing DNA which are less expensive such as that using sodium acetate. Thus, the sodium acetate method was evaluated in the extraction of *Mycobacterium ulcerans* DNA for detection by PCR. The objective of this work was to evaluate the performance of the sodium acetate technique in the extraction of *Mycobacterium ulcerans* DNA.

MATERIAL AND METHOD

Type, setting and study sites: The present study is prospective of validity of the tests which took place from January to April 2018. Within the framework of the surveillance of the BU in the DRC, the samples of various provinces were received at the National Institute of Biomedical Research (INRB) for analysis.

Two provinces were concerned by this study namely Kinshasa (Ngaba, Limete, Bumbu and Kasa-Vubu) and Kongo Central (IME-Kimpese).

Study population: Samples of suspected BU patients with skin lesions were collected from the above-mentioned sites. Biological analysis (ZN and PCR) were performed at INRB. Included in the study were any samples from patients suspected of Buruli ulcer who had consulted different medical facilities.

Data collection: Upon receipt of the sample, a laboratory number was assigned to each sample and recorded in the laboratory notebook. The samples were accompanied by a card in which all the patient's information was mentioned, namely age, sex, address, type and site where samples were taken.

Analysis of samples in the laboratory: Three biological tests were carried out in the Mycobacteria laboratory for confirmation of cases Ziehl-Neelsen, PCR using Sodium Acetate and PCR using the Maxwell Kit.

Direct examination after coloring Ziehl Neelsen: After preparation of the suspension, a drop of inoculum was spread on a dried slide and fixed with a burner (Aubry *et al.* 2017). The cold method was used to stain the slides and this method consisted of staining the smears with concentrated Ziehl fuchsin for 30 minutes, bleaching with the acidic alcohol for 3 minutes and recolouring with methylene blue for one minute. The reading was done at Objective 100X to visualize the bacilli.

The molecular procedure: Two methods were used for the extraction of Deoxyribonucleic Acid (DNA), the first being the automated method using the Maxwell kit (Promega) and the second being the manual method using Sodium Acetate. Before any extraction, a membrane lysis for each sample suspension was carried out beforehand (Aubry *et al.*2017; Affolabi *et al.*2012; De souza *et al.* 2012; Bretzel *et al.* 2007). For a quantity of 200 µL of suspension in a microtube, 200 µL of lysis buffer and 10 µL of proteinase K were added and these mixtures were placed in the wells of the thermoblock set at 60 ° C. for incubation for 16 hours (Affolabi *et al.*2012; Bretzel *et al.* 2007). The Lysat thus obtained could be extracted by the two methods mentioned above. It should be noted that for these extraction steps, two controls have been arranged (positive and negative). Kit includes cartridges, elution cuvettes (blue tube), plungers and elution buffer. The following steps were followed to extract the DNA: each cartridge as well as the blue cuvettes were numbered with the corresponding number of microtubes containing the predigested sample, the cover covering each cartridge was cautiously removed to ensure that there is no more plastic covering the holes. 400µL of the predigested tube contents were pipetted into the first hole of the cartridge corresponding to the sample, the divers were placed in the seventh hole of the cartridge, while taking care to touch only the head of the plunger and the elution bowl was touched making sure not to touch its interior (Affolabi *et al.*, 2012; Strientra *et al.*, 2010; Bretzel *et al.*, 2007). Elution buffer 300 µL was pipetted into each cuvette and the Maxwell-16 machine was connected to the network behind a stabilizer and an inverter by pressing the green power button on the back of the machine then wait for the signal at the end of the work before opening the door and pushing the green validation button <Run / Stop> to start the analysis.

After 45 minutes, the machine signals the end of the extraction(1,8-9). The DNAs collected in the blue cuvettes are transferred to new sterile microtubes previously numbered and stored at -20 ° C (Affolabi *et al.*2012; Bretzel *et al* 2007; Strientra *et al.* 2010).

Manual extraction method with Sodium Acetate (CH3COONa): To 400µL of lysat were added two volume of 95% ethanol (800µL) and 3/10 volume of 3M sodium acetate (120 µL). The mixture was stirred and incubated at 20°C overnight. It was then centrifuged at maximum speed for 30 min., on cool rotor. After aspiration of supernatant, 500 µL of 70% ethanol was added to the pellet after aspiration of supernatant and the mixture centrifuged at maximum speed for 10 min. The supernatant was aspired and left open for few hours (Jemmali *et al.* 2007). Then 50 µL of elution buffer was added and vorttexed until dissolution of the pellet before storing at -20°C (Jemmali *et al.* 2007).

RT PCR (mix preparation, amplification and reading): The summary sheets were prepared for all the samples, including those from two extraction controls and the 3 standard amplification controls (the controls containing 25, 50 and 50,000 copies of DNA) without forgetting the quantity of the reaction mixture(Jemmali *et al.* 2007). In pre-PCR, the mix (reaction mixture) was prepared in a 1.5 mL microtube in which 12.5 µL of the Bioline Sensimix II Probe master mix enzyme, 0.5 µL of primers, 0.56 µL of probe were mixed. 0.56 of Rox and 7.8 µL of water of molecular biology (Jemmali *et al.* 2007; De Souza *et al.* 2012). The master mix thus obtained was distributed at the rate of 22.5µL in each well of the PCR plate corresponding to the samples as well as to the various controls (extraction and amplification controls). In the extraction room, 2.5µL of each DNA extract was added and the plate was sealed with an adhesive film and carried gently in post PCR and introduced into the ABI 7500 FAST Real machine -Time PCR System coupled to a computer (Affolabi *et al.* 2012, De Souza *et al.* 2012; Jemmali *et al.* 2007). The reading was performed by comparing either the amplification curves <Amplification Plot> or the Ct <Report> values of the different controls (extr +; extr-; standards) with those of the different samples.

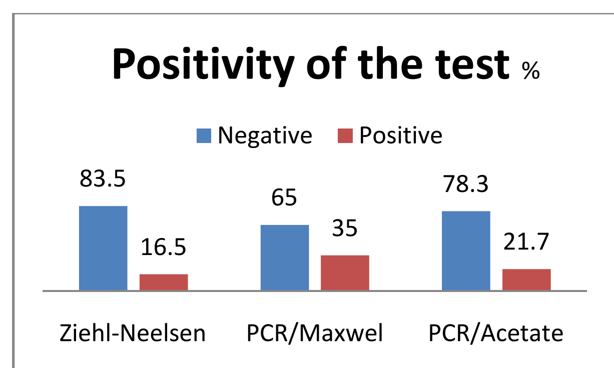

Table 1. Distribution of patients by age group

Age group	n	%
6months-5	5	5.2
6-16	12	12.4
17-27	19	19.6
28-38	7	7.2
39-49	16	16.5
50-60	14	14.4
61-71	8	8.3
72-82	5	5.1
≥83	11	11.3
Total	97	100

Ethical considerations

Patient data was well kept confidentially

Statistical Analyzes: The data recorded in the register was then transferred to the Excel software. They were analyzed using SPSS software version 20.0. Mean and standard deviation were calculated for the quantitative variables. The PCR results obtained after extraction of the DNA by the sodium acetate method were compared with those obtained by extraction of the DNA with the Maxwel method considered as a reference test. The results obtained by Ziehl staining were also compared to those obtained by PCR using the Maxwell method. Sensitivity, specificity, positive and negative predictive values as well as Kappa and Youden index were calculated. Ficher's exact test and Pearson's Chi-square were used as needed. The significance level was set at 5% and confidence interval (CI) at 95%.

RESULTS

During the study period 97 samples were recorded and analyzed. There were 39 males (40.2%) and 49 females (50.6%) and 9 patients (9.3%) sex were indeterminate (p = 0.067); the ratio being 0.8. The mean age of all patients was 38.32 ± 22.33 years with extremes ranging from 6 months to 88 years. As shown in table I , the age group 17-60 was more represented (57.7%), followed by the 6 month-16 years old group (17.6%) and the group ≥83 (11.3%). %). The others had lower proportions (8.3% and 5.1%). The distribution of patients by province shows that 79% of samples came from Kongo Central and 21% came from Kinshasa (p = 0.000). 85.6% of swabs were recorded followed by 12.4% of fine needle sampling and 2% of scrapings from scraping. The proportion of Ziehl positive compared to that obtained by Maxwell, shows a significant difference (p = 0.003). On the other hand, the same proportion of positive Ziehl compared to that of the Sodium Acetate method does not show a significant difference (p = 0.36). Comparing the positive PCR result using Sodium Acetate to that of Maxwell, the difference is significant (p = 0.038). The Smear Positivity by sex shows that 15.38% of smears were positive in male patients and 12.24% in female; p = 0.67. In contrast, 44.4% of smears of patients with indeterminate sex were Ziehl positive.

The positivity of the PCR using the sodium acetate method according to sex shows that 22.45% of *Mycobacterium ulcerans* DNA was detected in women against 15.38% in men; P = 0.404. The positivity of the PCR using the Maxwell method according to sex shows that 35.90% of *Mycobacterium ulcerans* DNA was detected in men against 30.61% in the women; P = 0.6. 17.1% of smears were Ziehl positive in age group ≥ 15 and 13.3% were positive in children <15 years; p = 0.72. PCR using the Sodium Acetate method detected 35.9% of *Mycobacterium ulcerans* DNA in the age group <15 years and 20% were in the ≥ 15 year age group; p = 0.87. On the other hand, the one using the Maxwell Kit detected 46.7% of *Mycobacterium ulcerans* DNA in the 0 <15 years age group and 32.9% in the ≥ 15 years age group; p = 0.31. Ziehl-Neelsen's positivity according to sample type shows that 18.7% smears made from swabs were Ziehl positive and 8.33% from the fine needle were positive. The positivity of the PCR by the sodium acetate method according to the type of sample shows that the highest proportion was observed with the swabs (22.89%) followed by that obtained by fine needle (16.67%). While the 100% scraping samples were negative. The positivity of the PCR using the Maxwell method according to the type of sample shows that the highest frequency was observed with the swabs (37.4%) and the samples taken by fine needle had a positive proportion of 25%. The scraped samples (100%) were negative.

DISCUSSION

Buruli ulcer is a debilitating disease requiring early management to minimize suffering, disability and socioeconomic impact. This study was initiated to determine the contribution of the sodium acetate method in the extraction of *Mycobacterium ulcerans* DNA for detection by PCR compared with that using the Maxwell Kit as a Standard. - Gold. The proportion of positive samples was 16.5% for Ziehl, 21.7% for sodium acetate PCR and 35% for that using the Maxwell kit. PCR by the sodium acetate method yields sensitivity, specificity, VPP and Kappa respectively of 52.9%, 95.2%, 85.7% and 0.53. In contrast, the sensitivity, specificity, VPP, Kappa, and Youden index for the Ziehl technique were 47.1%, 100%, 100%, 53.6%, and 0.47, respectively. Ninety seven (97) samples were analyzed during the study period from 40.2% of men (39), 50.6% of women (49) and 9.3% of indeterminate sex. But, the difference between the two sexes was not significant (p = 0.067).

The study reports that the age group 0 to 16 was the least concerned (17.5%) compared to the age group of 17 to 83 (82.5%). The study reports that 79% of samples came from Kongo Central Province whereas 21% came from Kinshasa Province with a significant difference (p = 0.000). This is because Kongo Central is part of the Buruli Ulcer Endemic Zone (Phanzu *et al*. 2009). We also noticed that 85.6% of samples were swabs. These results corroborate those of Busukayi and al. (2015) and Herbinger and al. (2009) that also show that most of the samples analyzed in laboratory are swabs (Affolabi *et al*. 2012). This is because patients consult at an advanced stage of the disease where ulcerations of different sizes are found (Aubry *et al*. 2017; Busukayi *et al*. 2015). Regarding the positivity of the tests, the study indicates that the highest proportion was recorded by PCR using the Maxwell Kit (35%) followed by PCR using sodium acetate (21.7%) whereas the Ziehl-Neelsen technique had only 16.5%. The frequency of 21.7% obtained by PCR using sodium acetate for extraction of *Mycobacterium ulcerans* DNA is

Table 2. Sensitivity, specificity of different tests used

Tests	Sensitivity% (IC95%)	Specificity% (IC95%)	VPP% (IC95%)	VPN% (IC95%)	VG% (IC95%)	L	Kappa	Youden
Ziehl-Neelsen	47.1 (29.78-64.87	100(94.31-100)	100(79.41-100)	77,78(67.17-86.27)	81.4 (72.27-88.62)	Infinite	0.54	0.47
PCR/Acetate	52.9 (35,13-70.22)	95.2 (86.71-99)	85.7 (63.66-96.95)	78.95 (68.08-87.46)	80.4 (71.1-187.78)	0.49	0.53	0.48

higher compared to that obtained by Ziehl-Neelsen (16.5%) with a difference not significant (p = 0.36). On the other hand, the Ziehl positive results compare to those obtained by PCR using the Maxwell Kit shows a significant difference (p = 0.003). These results show the limit of the Ziehl technique to detect cases and this weakness has been demonstrated by several authors. (Aubry *et al*. 2017; Busukayi *et al*.2015). On the other hand, when comparing the results observed between the two PCRs (Maxwell and Sodium Acetate), a significant difference is observed (p = 0.038). This shows that PCR using the Maxwell Kit is more cost-effective than using Sodium Acetate for the extraction of *Mycobacterium ulcerans* DNA. Nevertheless, this method can be used in the absence of Maxwell's Kit because of its high positivity compared to Ziehl. However, the proportion of 35% obtained in this study is lower than that of 75%, observed by Phanzu and al. (2009) in a study on the contribution to the improvement of control of Buruli ulcer in the territory of Songololo (Kongo Central).

While the proportion of 35% found in this study is greater than that obtained by Busukayi and al. (2015). The difference in frequency can be explained by the size of the sample (97, 24 and 175). The frequency of 21.7% obtained by PCR using sodium acetate for extraction of DNA is higher than that obtained by Ziehl-Neelsen (16.5%) with a non-significant difference (p = 0.36). The limit of the Ziehl technique has been demonstrated by several authors by its weakness to detect fewer cases (Aubry *et al*. 2017; Busukayi *et al*. 2015). Comparison of the Ziehl positive results with those obtained by PCR using the Maxwell Kit shows a significant difference (p = 0.003). On the other hand, when comparing the results observed between two PCR (Maxwell and Sodium Acetate), we notice a difference that is also significant (p = 0.038). PCR using the Maxwell Kit is better than that using sodium acetate method. Nevertheless, sodium acetate method can be used in the absence of Maxwell Kit. The positivity of the tests according to sex showed a significant difference for the Ziehl (15.38% vs 12.24% in favor of men, p = 0, 67), PCR Acetate (15.38% vs 22.45% in favor of women, p = 0.404) and Maxwell PCR (35.90% vs 30.61%, p = 0.6). This is consistent with literature showing that Buruli ulcer affects both men and women in the same way (Aubry *et al*. 2017). There was no significant difference between the positivity of the tests and the two ages (<15 and ≥ 15 years). For ZN, P = 0.72, PCR Acetate; p = 0.87 and Maxwell PCR; p = 0.31. This is contrary to the literatures which show that the under-15 age group is the most affected by Buruli ulcer because it is constantly exposed by their play activities conducted around and in water sites (Phanzu *et al*. 2009). Regarding the positivity of the tests according to the samples, our study did not show a significant difference. This is at odds with some work that showed that this positivity depended on the nature of the specimen, especially the authors biopsy had a high rate of positivity that was not found in our study (Busukayi *et al*. 2015; Herbinger *et al*. 2009). Regarding the contribution of the method used compared to the gold standard PCR using the Maxwell kit, it is noted that PCR using sodium acetate for the extraction of *Mycobacterium ulcerans* DNA has a sensitivity, specificity , PPV, Kappa and Youden index respectively at 52.9%, 95.2%, 85.7%, 52.8% and 0.53. These results illustrate that the test is less sensitive, specific, more or less reproducible and less effective because the index is far from approaching 1. However, this method can be used in resource-limited countries if Maxwell Kit is missing. Maxwell requires financial means for its acquisition. Sodium acetate method is manual, simple and easy to use. The study reports a low sensitivity (47.5%) for the Ziehl. This sensitivity, although low, is higher than that of 12.5% observed by Busukayi and al. in 2015. However, it is lower than the 62% obtained by Phanzu and al. in 2009. Herbinger and al. in 2009 reported a sensitivity of 56.7% using as Gold Standard, histopathology (Busukayi *et al*. 2015).

Limit of the study: The small simple size: analysis concerned only two provinces and only a few structures. It should be noted also the nature of the samples which is not exhaustive.

Highlights of the study: The survey is reinforced by its prospective nature and the strength of the results is supported by the quality of the statistical tests which made possible to determine the contribution of various tests used.

Conclusion

This study shows that the Maxwell method detects more *Mycobacterium ulcerans* DNA than the less sensitive but specific sodium acetate method. **However this method of sodium acetate can be used in the absence of Maxwell kit for DNA extraction although the Youden index is low.** Men and women were affected in the same way, and the under-15 age group was affected in the same proposition as the 15-plus age group. Positivity was higher in the swabs than in the other samples.

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