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Full Length Article

Maximizing microscopy as a diagnostic tool in peripheral health centres of BU endemic areas in Ghana

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ABSTRACT

Background: Buruli ulcer (BU) disease, a skin condition caused by *Mycobacterium ulcerans* (*M. ulcerans*) is endemic in remote rural areas. Disease diagnosis on clinical basis alone can be misleading, requiring definitive diagnosis based on laboratory tests. Resource constraints in BU endemic areas make microscopy for the detection of acid fast bacilli (AFB) an important and useful method. It is rapid, user-friendly, convenient and cheap. Despite its usefulness, its performance is relatively low. This study investigated modifications of the current method aimed at improving its performance. Forty (IS) 2404 polymerase chain reactions (PCR) positive BU samples were processed by eight physical (centrifugation and overnight sedimentation) and chemical (phenol ammonium sulphate and sodium hypochlorite) modifications of the current direct method. Assessments were based on standard AFB evaluation coupled with in house criteria; positivity (P), clarity and contrast (C) release of bacilli from specimen (R). Overall AFB positivity rate was 64% (409/640). Each protocol had 80 smears. The percentage positivity (P) for the conventional method was 58% (46/80) smears. The highest positivity rate of 57/80 (%) was by protocol 7 (5% phenol in 4% ammonium sulphate (PhAS) and concentrated by overnight gravitational sedimentation). The least positivity rate at 35% (28/80) was by protocol 1 (smears from direct application of swab tips). The differences in performance between the two chemical tested; 5% phenol in 4% ammonium sulphate (PhAS) and 3.5% NaHOCl was significant ($p < 0.05$). The differences between the two physical methods were however not significant ($p > 0.05$). This study concluded that BU samples treated with a solution of 5% phenol in 4% ammonium sulphate and concentrated by either centrifugation or overnight sedimentation is useful for maximizing AFB detection by bright field microscopy. This can be useful in rural health facilities with resource constraints.
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Introduction

Mycobacterium ulcerans (*M. ulcerans*) causes Buruli ulcer (BU) disease which clinically manifests as nodules, papules, edemas and ulcers [1]. The condition has been reported in over thirty countries worldwide including Ghana [2,3]. BU disease occurs in remote rural water logged areas where access to healthcare is difficult [4]. Currently, the management of the condition relies on an 8 weeks administration of rifampicin and streptomycin [5]. In BU endemic communities, other skin conditions may exhibit disease syndrome similar to Buruli ulcer lesions [6]. This makes disease diagnosis based on clinical judgments alone unreliable. This observation was corroborated in a study by Etuaful et al., where an estimated 40% of clinically diagnosed BU cases turned out to be conditions other than BU [7]. This could lead to misapplication of antibiotics and its repercussions on public health. In view of this, The World Health Organization (WHO) emphasizes on the need to administer antibiotics only to laboratory confirmed BU cases [8]. Compliance with these recommendations can be challenging particularly in health centres located in endemic communities with resource constraints [9].

Microbiological methods recommended for laboratory diagnosis of BU include (i) the detection of Zeihl–Neelsen (ZN) stained acid fast bacilli (AFB) from BU samples by bright field microscopy (ii) culture of viable *M. ulcerans* isolates on Lowenstein–Jensen (L–J) media, (iii) the detection of *M. ulcerans* DNA by insertion sequence 2404 (IS2404) and (iv) histopathological analysis of biopsied tissue. BU samples positive for the presence of *M. ulcerans* by two of the laboratory methods is confirmatory of BU disease [8].

Implementation of these recommendations is however challenging; IS2404 PCR, the most sensitive and rapid method is costly, requiring special skills and input economically beyond the reach of health facilities in most BU endemic communities. Culture, the gold standard is slow, requiring a minimum of 8 weeks to obtain results and cannot be used in routine diagnosis [10]. Histopathological analysis is also sensitive but too cumbersome to be routinely used in the laboratories of health facilities of rural BU endemic areas [2].

Microscopy for the detection of AFB from Z–N stained smears is however simple; not requiring special skills. It provides presumptive information on status of AFB in sample. In addition, it is comparatively cheaper, rapid and user-friendly [11]. It also represents a convenient method which can easily be used in health centres of rural endemic communities with limited resources [12]. The sensitivity of this method is relatively low (<40%) and can potentially generate false negative cases [13]. Limiting factors include sample processing and AFB detection by microscopy [14]. Effective detection depends on the quantum of detectable bacilli in the sample. Studies have shown that sample concentration by centrifugation or overnight gravitational sedimentation have relatively improved the rate of AFB detection [15,16]. The detection of AFB attached to host tissue is difficult to view under bright field microscopy; studies have proved the usefulness of pretreatment of samples [17] particularly with lyzing chemical agents, which include hypochlorite, phenol ammonium sulphate and chitin with lyzing properties. These chemical agents effectively cause a detachment of *M. ulcerans* from infected host which can interfere with observation [18–20].

Differences between stained AFB and field of observation, a phenomenon variously described as clarity and contrast is an important influencing factor that could affect smear reading [21]. The improvement of these factors can greatly improve smear reading and also reduce episodes of false negative as reported in *M. ulcerans* microscopy. Sample processing could also affect outcome of staining.

This study investigated smear processing method aimed at improving on the conventional method of ZN-stained AFB by bright-field microscopy using IS2404 PCR confirmed BU samples.

Materials and methods

Study setting

The study was prospectively carried out between October 2010 and April 2012 in Paakro in the Akuapem South and Asuboi in the Suhum-Kraboa-Coaltar districts, in the Eastern region of Ghana. The Eastern region of Ghana occupies an area of 19,323 square kilometers with twenty-one administrative districts including the two study areas. Parts of the Eastern region have been identified by the Ghana health service as areas endemic for Buruli ulcer disease [22].

Study samples were part of specimens obtained from 135 clinically diagnosed BU cases based on WHO standards for the clinical diagnosis of BUD [23]. Ethical approval for this study had been sought from the institutional review boards (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR) of University of Ghana and the Ghana Health Service (GHS) of Ministry of Health. In addition, demographic information had been sought from eligible consenting persons with the help of structured questionnaire. Sample analysis took place in the Noguchi memorial institute for medical research (NMIMR) and the Public Health Reference Laboratory (PHRL) of Ministry of Health, Korle-Bu, Ghana. Samples used for this study were selected from samples previously confirmed by IS204 PCR.

Collection and processing of BU specimens

Sample collection

Samples were obtained as swabs and fine needle aspirates from Buruli ulcer lesions of infected cases exudates from ulcerative lesions were obtained by circling the undermined portions and crevices of the lesions with cotton swabs, whilst aspirates from central portions of pre-ulcerative lesions were collected with needle and syringe [24].

Samples were transported to the laboratory as swabs in 15 ml capacity receptacles and aspirates in 2 ml of sterile phosphate buffered saline.

Sample processing

BU samples were processed as sample suspensions by (i) agitation of swab tips in 2 ml of sterile distilled water with 10–15

spherical glass beads to facilitate release of exudates from tip of swab as described [25]. Fine needle aspirates were flushed in 500 μl of sterile phosphate buffer [26].

Laboratory confirmation of BUD by IS2404 specific PCR

DNA extraction

DNA was extracted from 500 μl of BU sample suspension by the QIAamp DNA extraction minikit (Qiagen, Hilden, Germany) as per instructions of manufacturer. In brief, protein in sample was denatured by agitation with20 μl of proteinase K. The cells in BU sample were lyzed to release DNA by agitation in 400 μl lysis buffer. Sample mix was incubated at 56 °C for 30 min to enhance activity. DNA extraction was done in two batches of 400 μl of absolute ethanol through spin column filtration by centrifugation at 6000*g*. Extracted DNA was washed in two batches of washing buffer provided in kit. DNA was eluted in 150 μl of the elution buffer also provided in kit. Extracted DNA was stored at −20 °C [27].

A cock tail of extracted DNA sample was prepared by the QIAGEN PCR kit as recommended by the manufacturer. Briefly 4 μl of extracted *Mycobacterium* DNA was amplified in 20-μl volume PCR reaction mix consisting of (1_Taq PCR buffer, de-oxynucleoside triphosphates (0.2 mM each), 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), each primer at a concentration of 0.5_M (represented in the PCR QIAGEN kit as master mix and Q solution in addition to 1 μl of forward Primer pMU1 and 1 μl of reverse pMU2, in addition to10 μl of Master Mix solution.

Prepared *M. ulcerans* DNA was amplified in a thermo-cycler (Applied Biosystems thermal cycler) under the following conditions staring with de-naturation at 95 °C for 5 min. PCR was performed for 40 cycles at 94 °C for 60 s, 66 °C for 60 s and 72 °C for 60 s. PCR reaction was terminated at 72 °C for 10 min. PCR products were trans-illuminated as bands (amplicons) after electrophoresis of PCR products in 2% agarose gel incorporated with ethidium bromide. The bands were estimated in comparison with 1-kb size markers which run concurrently with negative and positive controls [28].

Modified smear preparation methods for the detection of AFB by bright field microscopy

BU sample processing for AFB smear microscopy

Forty BU samples previously confirmed by the IS2404 PCR as positive samples were selected for this investigation. Each sample was processed by eight protocols including the conventional smear preparation method. The other seven methods were modifications of the conventional method. All smears were prepared in duplicates making a total of eighty smears per protocol.

Conventional direct smear method

Smears were prepared from 20 μl BU sample dropped centrally on a clean slide. The sample was spread in a proscribed portion of the slide and allowed to air-dry. Smears were stained by the Ziehl–Neelsen method as described [29].

Modified methods for AFB smear microscopy

Direct smear from swab: Smear was prepared by the direct application of the swab tip on the slide directly from swab exudates from lesion (in-house procedure).

Concentrated smear method 1: 100 μl of processed BU sample was concentrated by centrifuging sample and discarding 80 μl of sample. Smear was prepared from 20 μl of sediment [16].

Concentrated smear method 2: 100 μl of BU sample was concentrated by leaving sample overnight on a flat surface. 80 μl of supernatant was discarded and a smear was prepared from 20 μl of sediment [12].

Sample processing by HOCl method

Concentration by centrifugation: Equal volumes of 3.5% HOCl and BU sample were vortexed in a tube and allowed to stand for 10 min. This was topped up to 1000 μl level, thoroughly mixed and centrifuged at 2500 for 15 min. Smears were prepared from sample sediment after the supernatant had been discarded [18,30].

Concentration by overnight sedimentation: Sample processed by the HOCl method was concentrated by overnight sedimentation method. Smears were prepared from 20 μl sedimented sample after discarding the supernatant [31].

Sample processing by PhAS method

Concentration by centrifugation: 100 μl 5% phenol/4% ammonium sulphate was added to 100 μl and vortex mixed. This was allowed to stand for 9 min after 1 min of vortex mixing. The mixture was topped up to1000 μl with sterile distilled water. A smear was prepared from 20 μl of sediment after centrifugation at 2500 for 15 min and discarding the supernatant [32].

Concentration by overnight sedimentation: Samples were allowed to stand overnight on a flat surface. The supernatants were removed and smears prepared from the sediment as per methods (Vasanthakumari, 1988). All smears were stained by the Ziehl–Neelsen technique for AFB detection after heat fixing as described [19].

The smears were examined and evaluated by scanning at 1000× magnification under oil immersion with a bright field microscope. The AFB were quantified in accordance with published standards and further evaluated based on the specified in house evaluation criteria [33].

Evaluation criteria

Evaluation of the samples was based on criteria generated in-house; modifications based on standard evaluation criteria for AFB detection in Mycobacteriology [13] (i) number of AFB per 100 fields (P), (ii) clarity plus contrast: indicating the extent of brightness in field (C), and (iii) release of AFBs from the host material (R) for easy visibility after ZN staining and examination by microscopy under oil immersion at 1000×.

The maximum score per criteria was 4 whilst the minimum score was 0. The expected maximum rating per test was 12 and the total number of tests (*n*) was 80. The expected maximum score per protocol was therefore 960 (80×12).

BU samples, processed based on modified protocols of the conventional acid fast bacilli adapted as reported by these studies [19,34,16] (AFB) procedures were analyzed based on standard criteria for evaluating AFB [13].

Statistical analysis

Data from study was entered in Microsoft Excel (MS Excel) and analyzed with STATA 11 (Strata Corp, College Station, TX) to address the objectives of the study. Data description involved estimating totals, means, frequencies, ranges and prevalence rates of the study variables. Significant differences, associations and interrelationships of the variables were also assessed at a level of $p = 0.05$. The performance of the various smear preparation methods were evaluated based on the level of positivity, clarity and contrast and release of AFBs from host material. These were scored and ranked based on AFB detection criteria. The expected total score for a given evaluation criteria was 4 per test and 12 for 3 criteria. A high score was indicative of the performance of the method applied (protocol).

Results

Detection of ZN stained AFB by bright field microscopy and modified methods

Forty IS2404 PCR positive BU samples, prepared as smears in duplicates ($2 \times 40 = 80$ smears) per protocol made a total of 640 slides per 8 protocols.

Protocol performances based on set criteria (i) (*P*) Number of AFB per 100 field's, (ii) *C* (Clarity plus contrast; indicating the extent of brightness in field) and (iii) *R* (Release of AFBs from the sample matrix or host material) for easy visibility after ZN staining and examination by microscopy under oil immersion at 1000× is indicated in Table 1.

Maximum score per criteria = 4; minimum score = 0.

Expected maximum score per criteria/protocol = 320.

Total score for 3 criteria per protocol = 960.

Total scores from 8 different protocols are presented in Table 1. The total scores obtained by the protocols per criteria ranged from 65 to 204 for positivity (P). The highest cumulative score was by Protocol 7 representing sample treatment with 5% phenol in 4% ammonium sulphate and concentration by gravitational sedimentation at. The lowest was smears from direct application swab on slide from lesion. Protocol 7 scored the highest (score = 306) for clarity and contrast. Whilst the lowest was scored by protocol 6 representing sample processed with 3.5% sodium hypochlorite and concentration by centrifugation. The score range for clarity and contrast ranged was 203–306. Score for release AFB released from host material was between 86 and 175. The highest score point was by protocol 7 whilst the least was by protocol. Estimated total score for all evaluation criteria set for all the protocols were between 360 and 686. Overall, the best performance was by samples treated with 5% phenol in 4% ammonium sulphate (PhAS). Details on the performances of all tested protocols are contained in Table 1. Compable statistical analysis estimated that the observed differences between the modified protocols and the standard method for AFB detection from BU specimens were all significant ($p < 0.00001$).

Discussions

This study represents an in-depth investigation of procedures potentially capable of improving AFB smear microscopy for BU samples. To the best of our knowledge, no studies regarding AFB microscopy using chemical agents including phenol ammonium sulphate (PhAS) and sodium hypochlorite (household bleach) are available in literature for MU studies, and a few have however been reported on *Mycobacterium tuberculosis* (MTB) [32,18,19,35]. BU samples processed with PhAS and concentrated by overnight gravitational sedimentation performed best (cum. score = 686; sensitivity = 97%). Equally favorable performances have been reported by others in MTB studies [19] (sensitivity – 85% sensitivity; specificity 83%). In addition, this study reported performance of PhAS sample smears as being in comparatively clearer with good contrast. PhAS of mordant effect and its ability to precipitate protein in solution [36]; this effect could enhance the aesthetics of the smear. This observed phenomenon has been

Table 1 – Comparative analysis of smear preparation protocols (conventional and modified) N = 640.

Smear preparation protocols $n = 80$	Performances of various protocols scores per evaluation criteria					*p*-Value
	Positivity (P) 320	Clarity/contrast (C) 320	Release from host material (R) 320	Total 960*	(%) score	
1	65	209	86	360	37.5	0.0001
2[!]	162	210	100	472	49	Std
3	163	229	126	518	54	0.0700
4	182	232	136	550	57	0.0010
5	98	185	147	430	45	0.1330
6	80	170	126	376	39	0.001
7[#]	205	306	175	686	71	0.0000
8	176	273	213	662	69	0.0000

Key: Smears prepared from BU samples processed by the protocols indicated: 1 = direct from swab, 2 = direct from sample suspension, 3 = sample concentrated by gravitational sedimentation, 4 = sample concentrated by centrifugation, 5 = 3.5% hypochlorite and gravitational sedimentation, 6 = 3.5% hypochlorite and centrifugation, 7 = 5% phenol in 4% ammonium sulphate plus gravitational sedimentation, 8 = 5% phenol in 4% ammonium sulphate plus centrifugation.

* = (positivity score + clarity and contrast score + release from matrix), n = total number of tests for each protocol = $40 \times 2 = 80$, [!] = conventional, [#] = $m \times n$ = best protocol.

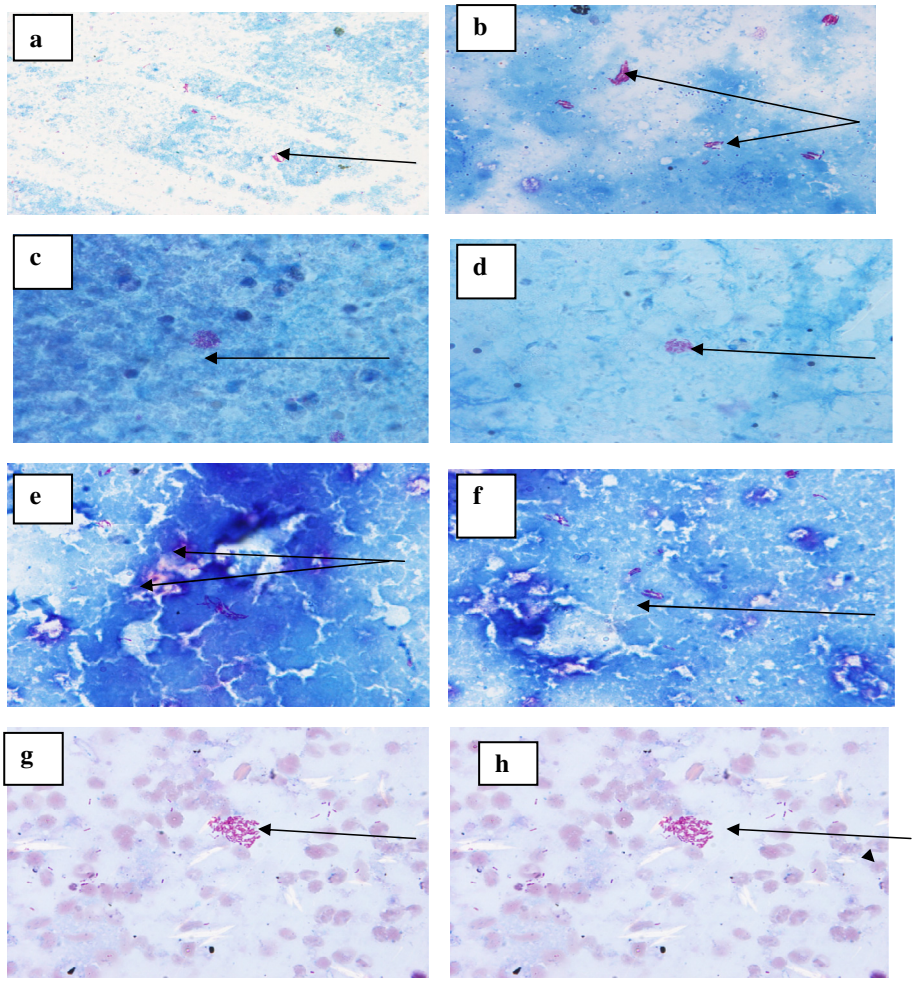

Figure 1 – (a–h) ZN stained smears of modified procedures on AFB detection. Figures (a–h) shows micrographs of various smear preparation protocols used (a) ZN stained smear prepared directly from swab. (b) Smear from BU specimen suspension. (c) Smear prepared from sedimented BU sample suspension. (d) Smear prepared from centrifuged BU sample suspension. (e) Smear from hypochlorite treated BU sample concentrated by gravitational sedimentation. (f) Smear from hypochlorite treated BU sample concentrated by centrifugation. (g) Sedimented (gravitational) phenol ammonium sulphate (PhAS) treated. (h) Sedimented (centrifugation) phenol ammonium sulphate (PhAS) treated.

reported by Singhal et al., also indicating the concentration effect and the formation of floccules with the AFB [32].

The direct application of swab for smear preparation was the worst performing method (cum. score = 374. Performance could be attributed to sampling and processing method. Method was an attempt to prepare smears directly from lesions under field conditions. This study demonstrated that smears from concentrated samples including with or without chemical treatment performed better than the direct AFB smear procedure. This is a widely observed phenomenon reported in MTB and MU studies [35,16,25,36].

The differences in performances between chemically processed samples concentrated by centrifugation and overnight gravitational sedimentation were not statistically significant ($p > 0.05$) in this study. Other studies in MTB however showed better performance for centrifuged samples [37]. The difference in performance between PhAS and HOCl processed samples was significant ($p < 0.00$). The influencing factor could be attributed to the inherent properties of the chemical agent used in these protocols. The differences were not only indicated by the scored ranks but by the micrographs of the prepared smears. Micrographs of smears prepared from PhAS showed clarity in the field of view, interspersed with elements which appeared as polka-dots. Selvakumar et al., working on sputum similarly reported that in addition to the fact that sputum samples treated with phenol ammonium sulphate had sensitivities almost similar to that of the direct method, it had several advantages including safety in handling, relatively shorter periods required for reading and though Collins et al. had reported that smears were washed off [19,33] it remained intact in Selvakumar study. Similar observations were made in this study where the smears remained intact in comparison to that treated with hypochlorite. In the case of smears processed with hypochlorite, portions of the field appeared relatively darker and visibly fissured as compared to the PhAS processed smears [38,30,11]. In this study,

samples treated with hypochlorite before various concentration methods did not perform as well as the other protocols, even though others had reported on the method improvement after bleach treatment [18]; subjective outcomes of slide reading efficiency with bleach treatment have been reported in MTB studies [16,37,12]. In the present study, NaHOCl processed smears have portions showing dark fields, obscuring some bacilli, whilst empirical evaluation of performance in terms of clarity and contrast indicated good performance, effective cumulative evaluation of all three criteria however showed poor performance for hypochlorite. The observed difference in smear reading in terms of clarity, may be due to procedural differences; whilst this study used a concentration of 3.5% hypochlorite, other workers used 1% hypochlorite [18,39]. In a related study, Chew et al. showed that bleach sedimentation significantly decreased the number of acid fast bacilli visualised compared with conventional smears. The study also concluded that though smear made from bleach sedimentation was more rapid and the inter-observer agreement was lower than the conventional method, he also observed that strong AFB positive smears were misread as negative, partly explaining the contradictory reportage of bleach treated smears. Observed AFB seemed obscured within the smear (see Fig. 1e and f). This may probably explain the findings by Chew et al. that hypochlorite sedimentation seemed to have a ten-fold decrease in AFB present. (bleach sed. = 185; conv = 205) [34]. The only limitation to the use of the sedimentation method would be the delay in diagnosis due to the overnight sedimentation process, but the clarity of the slides produced by this method will reduce the slide reading period and so compensate for the delays. This challenge of delays can be surmounted with investigations into time series to optimize appropriate sedimentation periods possibly falling into a time range less than the stipulated overnight period. Results obtained from this study have shown that concurrently processing BU specimens by physical and chemical methods improved AFB detection. The sodium hypochlorite treated samples appear "washed off" after staining and so care should be taken when staining. This is in accordance with other findings including a systematic review of 83 publications on the subject which concluded that, compared with direct smears, concentration of sample after chemical treatment was better [34,15]. This is a convenient method for the diagnosis of *M. ulcerans* infection, particularly in endemic under-resourced communities where the lack of adequate infrastructure is a challenge. The findings from this study will also reduce some limitations associated with detection of AFB, especially with swab specimens from paucibacillary lesions. This will ultimately enhance disease control measures and public health. Increasing sensitivity of AFB detection by microscopy is important and should be a public health priority [20].

Conclusion

This study concluded that laboratory diagnosis of Buruli ulcer disease by AFB smear microscopy can be improved by applying simple chemical and physical procedures to the BU samples. This includes sample processing with a solution of 5% phenol in 4% ammonium sulphate and concentrating by gravitational sedimentation is useful. This is also a simple, convenient, cheap method that can easily be applied especially in rural endemic areas with limited access to costly sophisticated methods.

Conflicts of interest

All authors have no conflict of interest to declare.

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