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Hydrochloric vs. sulphuric acid in water for Ziehl-Neelsen staining of acid-fast bacilli

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SUMMARY

SETTING: Damien Foundation Bangladesh tuberculosis (TB) control projects.

OBJECTIVES: To compare 25% sulphuric acid in water (H_2SO_4) with hydrochloric acid in water (HCl) to differentiate acid-fast bacilli in sputum smears stained with 1% carbolfuchsin.

DESIGN: For 1 year, all 158 microscopy laboratories used either H_2SO_4 or 3%/6%/10% HCl for their routine work, alternating monthly between H_2SO_4 and HCl. Each month a sample of five smears per laboratory was rechecked blind. After recording qualitative staining aspects, all sample smears were restained before rechecking, using H_2SO_4 for destaining.

RESULTS: A total of 368 059 H_2SO_4 and 335 436 HCl smears were routinely read, yielding 7.2% positive or scanty results in both groups. Of these, 9492 were rechecked. There was no difference in false-negatives detected (0.66%, 95%CI 0.44–0.95 for H_2SO_4 vs. 0.68%, 95%CI 0.46–0.98 for HCl), but apparently there were more false-positives with H_2SO_4 (2.12%, 95%CI 0.92–4.14 vs. 0.28%, 95%CI 0.00–1.54, $P = 0.05$). Qualitatively, only 3% HCl yielded significantly inferior differentiation results.

CONCLUSIONS: HCl 6–10% in water can be recommended for Ziehl-Neelsen destaining above H_2SO_4. Diluting is easier and safer, and it may cause less confusion with false-positives during rechecking, including a restaining step.

KEY WORDS: tuberculosis; destaining; Ziehl-Neelsen method; acids; acid-fastness

IN OUR EXPERIENCE, almost all national tuberculosis control programmes (NTPs) use either acid alcohol (3% hydrochloric acid [HCl] in alcohol) or 20–25% sulphuric acid (H_2SO_4) in water for Ziehl-Neelsen (ZN) staining of acid-fast bacilli (AFB). Acid alcohol works easier; however, cost and sometimes also regulatory problems for procurement of the large amounts of alcohol required by a network may be prohibitive, and with practice virtually the same results can be obtained using H_2SO_4.[1] In the original ZN technique, H_2SO_4 25% in water had been preferred over nitric acid by Neelsen.[2] Hardly any studies comparing acids for decolourisation seem to have been published afterwards. However, even H_2SO_4 has its problems. The relatively large volumes needed and its highly corrosive nature render transport difficult and costly. Handling bulk quantities during dilution is difficult due to its heaviness and viscosity, besides the considerable heat generated. Environmental considerations could be taken into account as well.

We performed an operational study comparing H_2SO_4 with HCl in water. The study was initiated because of safety concerns regarding the handling of large amounts of H_2SO_4 by our reference laboratories' technicians, preparing staining solutions for the 158 microscopy laboratories of the Damien Foundation Bangladesh TB control project (DF). Moreover, HCl 10% had already been used out of necessity at a time when the NTP had erroneously procured HCl instead of H_2SO_4, with apparently excellent results. With this pre-knowledge, permission was obtained from the NTP and from the Ethics Advisory Group of the International Union Against Tuberculosis and Lung Disease to perform the study under routine conditions.

METHODS

During a full year (2007), all 158 DF AFB smear laboratories participated in the study, decolourising all their routine smears with either H_2SO_4 or HCl diluted in water. Alternating monthly with H_2SO_4, HCl was used first at 3%, i.e., 30 ml of fuming HCl 37% diluted to 1 l with distilled water. This was changed to higher concentrations (6% or 10%) after the first evaluations had showed qualitatively unsatisfactory results. All laboratories processed approximately equal numbers of smears with the two acids. However, for

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practical reasons the HCl variations were allocated to project areas with sometimes quite different workloads, resulting in unequal numbers processed with each variation. The ZN NTP guidelines were followed for smearing (3 cm × 2 cm, using disposable bamboo sticks), staining by hot ZN technique (at least 10 min using 1% basic fuchsin; 0.1% methylene blue counterstaining for 1 min) and reading (one length before declaring a smear negative at 1000× magnification). Destaining was always performed for 3 min, repeating as needed based on visual inspection. Staining solutions were prepared at the five DF project reference laboratories also responsible for rechecking external quality assessment (EQA), using chemicals provided by the NTP. Brands varied, but most often these were technically pure quality chemicals or certified biological stains manufactured in India.

After letting the oil drain off overnight, all smears were kept in boxes. As per routine EQA instructions based on international recommendations, a random sample of five smears was collected monthly and sent for rechecking by the local supervisor, noting the type of acid used on the list. Prior to rechecking, the controllers gave their standardised qualitative appreciation of AFB staining and background. EQA sample smears were then restained following the same routine procedure. However, all sample smears were now decolourised with H_2SO_4, as it could not be assumed that errors possibly provoked by the experimental HCl destaining would otherwise be visible to the controllers. Rechecking was blinded for the first controls, but not for the second control on discordant smears at the main DF reference laboratory, when only the origin of each result was hidden. As this was linked to specific calendar months and laboratories, controllers could not be blinded to the type of acid used in routine. Using the second controller result as the gold standard, errors were classified as high and low false-positive (HFP, LFP), high and low false-negative (HFN, LFN) and quantification error (QE), as per international guidelines.[3]

Summary AFB microscopy and rechecking of data entry were performed at project level using spreadsheet formats. These were exported to Epi Info 6.04d for analysis (Centers for Disease Control and Prevention, Atlanta, GA, USA). Pearson's χ^2 was used for comparison of proportions, with calculation of the 95% exact confidence intervals (95%CIs).

RESULTS

Of 703 495 ZN smears processed by the routine laboratories in 2007, 123 516 were decolourised with 3%, 161 680 with 6% and 50 240 with 10% HCl (total 335 436, Table 1). The remaining 368 059 smears were treated with 25% H_2SO_4. Although minor differences in the prevalence of positive or scanty positive smears were detected within the HCl group, its overall percentage was almost the same as with H_2SO_4 (7.16% vs. 7.18%). A total of 9492 smears were rechecked (4745 HCl and 4747 H_2SO_4 decolourised), including 7.76% with a positive or scanty positive result (7.59% for the HCl and 7.94% for the H_2SO_4). The variation of positive and scanty smear prevalence was larger comparing the different HCl concentrations (Table 1).

Table 2 shows the numbers of errors detected by rechecking. False-positive (FP) errors were very rare, with only one LFP for the HCl group (0.28%, 95%CI 0.00–1.54), and four LFP plus four HFP for H_2SO_4 (2.12%, 95%CI 0.92–4.14). The difference was borderline significant ($P = 0.05$). Virtually the same proportion of false-negative error (FN) was detected for the HCl group as for H_2SO_4 (0.68% vs. 0.66%), corresponding with sensitivities of respectively 92.3% and 92.9% (non-significant). Within the HCl group the differences between FN percentages (range 0.48–0.86%) as well as sensitivities (range 91.2–94.2%) were small and non-significant. The proportion of QEs was low and very similar for HCl (1.1%) and H_2SO_4 (0.8%).

Table 2 also shows an analysis of the qualitative appreciation of the smears by the controllers. Neither AFB colour nor background decolourisation differed significantly between HCL and H_2SO_4 smears, with respectively 89.4% and 89.9% and 93.5% and 95.4% rated positively. However, within the HCl group the 3% concentration scored less well for AFB (83.5%, $P = 0.04$) as well as background (87.7%, $P < 0.0001$) compared to both the 6% and 10% concentrations.

Table 1 Number of smears and per cent positive or scanty results in routine work vs. rechecking samples by destaining reagent used in routine

	Routine smears				Rechecked smears			
Destaining reagent	Total *n*	Positive *n*	Scanty *n*	Positive or scanty %	Total *n*	Positive *n*	Scanty *n*	Positive or scanty %
3% HCl	123 516	7 118	1310	6.82	1560	78	19	6.22
6% HCl	161 680	9 727	2111	7.32	2412	173	25	8.21
10% HCl	50 240	3 194	547	7.45	773	59	6	8.41
Any HCl	335 436	20 039	3968	7.16	4745	310	50	7.59
25% H_2SO_4	368 059	22 136	4273	7.18	4747	326	51	7.94
All smears	703 495	42 175	8241	7.17	9492	636	101	7.76

HCl = reagent grade hydrochloric acid diluted in water to 3%, 6% or 10%; 25% H_2SO_4 = reagent grade sulphuric acid diluted in water to 25%.

Table 2 Results of rechecking and qualitative smear evaluation by destaining reagent

	3% HCl		6% HCl		10% HCl		Any HCl		25% H_2SO_4	
Errors detected	*n*	% (95%CI)	*n*	% (95%CI)	*n*	% (95%CI)	*n*	% (95%CI)	*n*	% (95%CI)
HFP	0		0		0		0		4	
LFP	0		1		0		1		4	
Any FP	0	0.0 (0.00–3.73)	1	0.51 (0.00–2.78)	0	0.0 (0.00–5.52)	1	0.28 (0.00–1.54)	8	2.12 (0.92–4.14)
HFN	4		3		1		8		5	
LFN	3		16		3		22		24	
Any FN	7	0.48 (0.19–0.98)	19	0.86 (0.52–1.34)	4	0.56 (0.15–1.44)	30	0.68 (0.46–0.98)	29	0.66 (0.44–0.95)
Sensitivity, %	93.3		91.2		94.2		92.3		92.9	
QE	1	1.0 (0.0–5.6)	3	1.5 (0.3–4.4)	0	0 (0.0–5.5)	4	1.1 (0.3–2.8)	3	0.8 (0.2–2.3)
AFB well stained, %	83.5		92.7		88.5		89.4		89.9	
Background well destained, %	87.7		96.5		99.0		93.5		95.4	

HCl = reagent grade hydrochloric acid diluted in water to 3, 6 or 10%; 25% H_2SO_4 = reagent grade sulphuric acid diluted in water to 25%; CI = confidence interval; HFP, LFP, FP = high, low and total false-positive; HFN, LFN, FN = high, low and total false-negative; QE = quantification error; AFB = acid-fast bacilli.

DISCUSSION

In the ZN technique, after applying the primary stain the AFB must be differentiated from the background, and particularly from other bacilli. This requires the use of strong acids. Alcohol can be used as well, but with some practice it is not indispensable. Mokhtari et al. found no difference comparing H_2SO_4 20% in watery or alcoholic solution.[4] Alcohol as a final step has also been recommended for homogeneous staining, counteracting the beading that occurs with some brands of fuchsin stain.[5,6] Gabbett combined acid destaining and methylene blue counterstaining in one solution.[7] The Tan Thiam Hok technique uses a cold saturated primary stain (as used also by Kinyoun), with the Gabbett modification.[8,9] Although widely adopted, its liability to error has been reported, and Devulder contradicted Tan Thiam Hok's claim of a 10% proportional increase in sensitivity compared to ZN.[10,11]

It is sometimes said that H_2SO_4 makes it easier to find the AFB since it removes some of the background material from thick smears (S J Kim, personal communication). Few comparative studies between acids have been conducted. Experiments in Copenhagen involved several different destaining techniques (Gabbett, nitric acid, sulphuric acid and alcohol[7]). Methods with a single destaining step revealed more AFB than those with 3–4 steps alternating H_2SO_4 and alcohol; however, as the primary staining technique also varied, no firm conclusions could be drawn.[12] The choice between acid alcohol and watery acids thus depends mainly on local preference, in addition to the availability and cost of the large quantities of alcohol needed for an extensive microscopy network. Also, at 20–25%, H_2SO_4 is needed in large quantities, is costly to transport, and technicians may object due to its difficult handling and highly corrosive nature. HCl is easier to handle, less expensive, possibly less corrosive at these concentrations, and also environmentally more acceptable. However, as for H_2SO_4 decolouriser, abundant rinsing during staining is required to avoid damage to metal drainage pipes in the long term. The concentrated 'fuming' 37% HCl that is usually procured constitutes a health hazard, and should preferably be diluted in a fume hood or simple air extraction cabinet. Where such appliances are not available, performing the dilutions in the open air or procurement of commercially available diluted HCl always remains possible.

Our study was set up to compare the AFB differentiating activity of low-concentration HCl in water with the original 25% H_2SO_4 of the ZN technique. We used both acids during alternating months, processing very large numbers of smears under routine but favourable conditions in a multicentre study. The 3% HCl initially used was soon found to leave too much red in the background, and repeated destaining was required too often. The HCl concentration was then increased to 6% in some of the laboratories, or to 10% in the others.

Considering all HCl concentrations together, the same percentage of positive and scanty positive smears were reported as with H_2SO_4. Differences observed between the variations were small but attained statistical significance; however, geographic as well as secular bias must be assumed, apart from the very high totals leading to statistical significance of even small differences. Their meaning thus remains obscure.

Further evaluation was performed by rechecking the EQA of random samples at the reference laboratories for close to 5000 smears for each of the two acids. A comparison of the errors detected revealed no difference in false-negatives, and only very rare FP. However, the excess FP with H_2SO_4 reached borderline significance, and half of those were HFP. In our opinion, this may represent an artefact caused by loss of AFB from the smears during restaining, which could happen more easily with smears destained twice with H_2SO_4, if indeed this acid removes more material from the mucoid matrix.[13]

Qualitatively, good differentiation of the AFB from the background was achieved by all the acid variations

tried, except for HCl 3%. As differences between HCl 6% and 10% on qualitative as well as rechecking parameters were small and balanced each other, any concentration in this range might yield satisfactory results.

Our study has some limitations. The numbers of smears processed by acid variation differed considerably, and technicians were not blinded to the technique. This was caused by the nature of the study, embedded as it was in the daily routine work of 158 busy microscopy laboratories. We tried to avoid extra work and confusion by linking the type of acid used to a certain month and area. The high numbers of smears and laboratories, still reaching 50 000 smears and 68 laboratories even for the least used 10% HCl variation, may have safeguarded against important bias.

CONCLUSION

For the differentiation of AFB in the ZN technique, HCl in water at 6% to 10% concentration is equivalent to H_2SO_4 25%. HCl 6–10% can thus be recommended as a less costly, easier to handle and less corrosive alternative. Moreover, confusion between true false-positives and AFB lost from smears during restaining before rechecking might occur less often with this acid.

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RÉSUMÉ

CONTEXTE : Les projets de lutte antituberculeuse de la Fondation Damien au Bangladesh.

OBJECTIFS : Comparer une solution d'acide sulfurique (H_2SO_4) à 25% dans l'eau avec diverses solutions d'acide chlorhydrique (HCl) dans l'eau pour différencier les bacilles acido-résistants dans les frottis de crachats colorés à la carbolfuchsine à 1%.

SCHÉMA : Pour leur travail de routine au cours d'une année, l'ensemble des 158 laboratoires de microscopie ont utilisé soit H_2SO_4 soit HCl à 3%, 6% ou 10% pour leur travail de routine, avec une alternance mensuelle entre H_2SO_4 et HCl. Chaque mois, un échantillon de cinq frottis par laboratoire a été relu à l'aveugle. Après enregistrement des aspects qualitatifs de la coloration, tous les échantillons de frottis ont été recolorés puis décolorés avec H_2SO_4 avant le recontrôle.

RÉSULTATS : Au total, 368 059 frottis H_2SO_4 et 335 436 frottis HCl ont été lus en routine, avec un rendement de 7,2% de résultats positifs ou très faiblement positifs dans les deux groupes. Parmi ceux-ci, 9492 frottis ont été recontrôlés. Il n'y a pas eu de différence dans la détection de faux négatifs (0,66% ; IC95% 0,44–0,95 pour H_2SO_4 vs. 0,68% ; IC95% 0,46–0,98 pour HCl) mais apparemment, il y a eu plus de faux positifs avec H_2SO_4 (2,12% vs. 0,28% ; les IC95% étant respectivement 0,92–4,14 et 0,00–1,54 ; $P = 0,05$). A l'examen qualitatif, les résultats de différentiation sont moins bons uniquement dans les frottis décolorés à l'HCl 3%.

CONCLUSIONS : Il est préférable de recommander l'HCl à 6–10% dans l'eau plutôt que le H_2SO_4 pour la décoloration du Ziehl-Neelsen. Sa dilution est plus facile et plus sûre et il peut entraîner moins de confusion en ce qui concerne les faux positifs au cours du recontrôle qui comporte une étape de recoloration.

RESUMEN

MARCO DE REFERENCIA: Los proyectos de lucha contra la tuberculosis (TB) de la Fundación Damien en Bangladesh.

OBJETIVOS: Comparar la utilización del ácido sulfúrico al 25% en agua (H_2SO_4) y del ácido clorhídrico (HCl) en agua en la diferenciación de los bacilos acidorresistentes en muestras de esputo coloreadas con fuchina fenicada al 1%.

MÉTODOS: Durante un año, los 158 laboratorios de microscopia usaron ya sea H_2SO_4 o HCl al 3%, al 6% o al 10% en la práctica corriente, alternando mensualmente el ácido empleado. Cada mes, se verificaba de nuevo en forma anónima una muestra de cinco baciloscopias por cada laboratorio. Tras registrar los aspectos cualitativos de la coloración, todas las baciloscopias de la muestra se colorearon de nuevo antes de la nueva verificación, usando H_2SO_4 como decolorante.

RESULTADOS: En el trabajo corriente se utilizó el H_2SO_4 en 368 059 de las baciloscopias leídas y el HCl en 335 436 y se obtuvo un 7,2% de resultados positivos y positivos con un número escaso de bacilos en ambos grupos. De estos, 9492 frotis se verificaron nuevamente. No se encontraron diferencias en los negativos falsos detectados (0,66%; IC95% 0,44–0,95 con el H_2SO_4 contra 0,68%; IC95% 0,46–0,98 con el HCl), pero al parecer se presentaron más positivos falsos con el H_2SO_4 (2,12%; IC95% 0,92–4,14 contra 0,28%; IC95% 0,00–1,54; $P = 0,05$). Desde el punto de vista cualitativo, solo el HCl al 3% mostró resultados de diferenciación notablemente inferiores.

CONCLUSIÓN: Se puede recomendar el uso del HCl en agua al 6% o al 10% como decolorante en la coloración de Ziehl-Neelsen en lugar del H_2SO_4. Las diluciones son más sencillas y más seguras y pueden causar menos confusión con positivos falsos durante las verificaciones posteriores que incluyen una nueva etapa de coloración.