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Laboratory diagnosis of tuberculosis in a large pediatric hospital in Cambodia

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SUMMARY

SETTING: Tuberculosis laboratory in the Jayavarman VII Children's Hospital in Siem Reap, part of the Kantha Bopha Hospitals, the largest pediatric hospital complex in Cambodia.

OBJECTIVE: To determine the efficiency of on-site microscopy and rRNA amplification in children with a clinical diagnosis of tuberculosis (TB) and specimen sampling for culture.

RESULTS: From 1 July 2005 to 31 March 2006, 52 400 children were admitted to the hospital. Among these, 405 children had tuberculosis, including 91 (22.5%) laboratory-confirmed cases, or respectively 7.7 and 1.7 per 1000 admissions. Among cases confirmed by microscopy or rRNA assay, rRNA identified 91.2%. Among all culture-confirmed cases, rRNA identified 90.5%. Culture alone contributed 7.1% to all laboratory confirmed cases. The yield of culture from preserved specimens was not affected by shipment delay. For 97.4% of the children, the maximum turnaround time for the on-site laboratory result was 48 h.

CONCLUSION: Implementation of a mycobacteriology service in a referral hospital is feasible, as the molecular technique is highly efficient. Storage of specimen aliquots allows subsequent culture without loss of viability due to shipment delay.

KEY WORDS: microscopy; molecular techniques; children

DESPITE its small population size (14.5 million), Cambodia ranks among the 22 high tuberculosis (TB) burden countries, with an estimated incidence of close to 500 cases per 100 000 population.[1]

In a 2002 survey, the prevalence of bacteriologically confirmed pulmonary TB was 1.2%.[2] No reliable data on TB in children are available apart from a difficult to interpret tuberculin skin test survey, reporting an annual risk of infection with *Mycobacterium tuberculosis* of about 1% for the year 1995.[3]

The Kantha Bopha children's hospitals in Siem Reap and Phnom Penh constitute the largest hospital complex in Cambodia offering free services to all children in need. In 2010, more than 100 000 children were admitted and three quarters of a million attended the outpatient clinics.[4] By 2003, it was decided to strengthen the TB laboratory services with three main goals: 1) increase the yield of on-site laboratory confirmation; 2) evaluate diagnostic laboratory procedures as to their usefulness in clinical practice linked to clinical data from children with TB; and 3) establish a systematic collection of clinical *M. tuberculosis* isolates.

This article describes service implementation and summarizes basic patient characteristics and laboratory results.

MATERIALS AND METHODS

Study design

The primary project purpose was to strengthen laboratory proficiency in TB diagnosis and to provide results within 48 h after admission of a child with a clinical diagnosis of TB.

In any child suspected of having TB on admission based on clinical or radiographic findings, a clinical decision was made as to whether or not the child had TB. If the decision was 'TB', laboratory examinations specific for TB were systematically performed. A protocol ensured the standardized flow-schedule for examinations. Aliquots of specimens were kept for processing by culture. Microscopy and rRNA amplification were established in the Jayavarman VII Children's Hospital in Siem Reap, Cambodia, while culture, drug susceptibility testing (DST) and strain genotyping were provided by the Institute of Infectious Diseases, University of Berne, Switzerland.

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Phase 1: Technical set-up phase, 1 January 2004–30 June 2005

Within the laboratory complex in Siem Reap, a TB unit was put into operation. This comprised reconstruction issues regarding biosafety, logistics, elaborating methods and defining the flow of the investigation procedures, and teaching and training of the medical, nursing and laboratory staff.

Phase 2: Clinical investigation phase, 1 July 2005–31 March 2006

Every child suspected of TB on admission was enrolled and given a unique study identifier in addition to the standard unique identifier each child is provided with at first hospitalization. Imaging techniques were utilized at the discretion of the clinician. Treatment was initiated once a clinical diagnosis of TB was decided. An appropriate specimen was collected on the spot, followed by additional specimens to obtain at least three specimens on 3 consecutive days. Pertinent data were abstracted and recorded in a study register, supervised by specifically trained attending physicians.

Phase 3: M. tuberculosis *isolate collection phase, 1 April 2006–15 July 2008*

The objective of this phase was to enlarge the *M. tuberculosis* isolate collection. To reduce the workload resulting from specimen aliquoting and processing from all patients with a clinical diagnosis of TB, collection of locally positive specimens for culture in the collaborating laboratory in Switzerland was reduced to a single pre-defined day per week.

Specimen collection and processing

Specimens were to be processed within 48 h. Following concentration and decontamination, three aliquots were prepared: one for an rRNA assay, a second for microscopic examination, and a third was stored for later shipment to Switzerland.

Concentration and decontamination

The specimens were homogenized, decontaminated with sodium dodecyl sulphate-hydroxide, neutralized, and centrifuged at 4000× *g* for 20 min at +4°C, and then re-suspended in 1 ml of phosphate-buffered saline.

Microscopy

A 100 μl of the sediment were used for smear preparation and staining by the Ziehl-Neelsen technique.[5,6]

rRNA amplification and detection

Using the Gen-Probe Mycobacterium Tuberculosis Direct Test (MTD® Test; bio-Mérieux Suisse SA, Geneva, Switzerland), 450 μl were used for detection of rRNA following the manufacturer's instructions.

Sediment storage

The third aliquot was stored at 4°–8°C for up to 6 months after addition of the antimicrobial cocktail PANTA to 0.5% Middlebrook 7H10 agar (Difco, Detroit, MI, USA), and sent in batches to the collaborating laboratory in Switzerland for culture, DST and genotyping.

Culture

One slant of Löwenstein-Jensen medium containing pyruvate and one BacT/ALERT Mycobacteria Process bottle with Mycobacteria Antibiotic Supplement (bio-Mérieux Suisse SA) were inoculated with 0.2 and 0.5 ml of the sediments. Slants of the solid medium were incubated in the BacT/ALERT Microbial Detection System (bio-Mérieux Suisse SA) process bottles at 35°C in ambient air according to the manufacturer's recommendations. Incubation was performed for 8 weeks and inspected weekly for growth, although the mean time to growth with this system is reported to be shorter.[7,8]

Identification

Partial sequencing of the 16S ribosomal RNA (rRNA) gene was performed using the MicroSeq 500 16S rRNA Bacterial Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA). Sequences were submitted to the Ribosomal Differentiation of Medical Microorganisms database (http://www.ridom.de/). *M. tuberculosis* complex isolates were differentiated using the GenoType® MTBC assay (Hain Life Sciences GmbH, Nehren, Germany).

Electronic data base and analysis

Data were captured in an EpiData relational database (EpiData Association, version 3.1, Odense, Denmark, freely available at http://www.epidata.dk). The primary file contained clinical and demographic patient information to which as many records as there were specimens with laboratory information for the child were related. Data on DST, spoligotyping and mycobacterial interspersed repetitive units-variable number tandem repeat typing available in a spreadsheet were merged with the relevant EpiData file. Data entry was constrained by a set of rules for legal values, but no double-entry for validation was pursued. Data were analyzed using EpiData Analysis (version 2.2.1).

Ethics

No formal informed consent was obtained since the study involved clinical isolates obtained during routine diagnostic work.

RESULTS

From 1 June 2004 to 31 July 2008, 595 children with TB were enrolled, with a total of 1516 specimens (2.5 on average per child). Each child had at least one specimen available: 170 children had only one, 23 had two, 322 had three, and 80 had four to seven specimens. Each specimen was examined by at least one,

Table 1 Tuberculosis cases enrolled in the study by study phase, and type and result of laboratory examination. Number of children and number of examinations of each type (microscopy by the Ziehl-Neelsen method, rRNA amplification, and culture), Kantha Bopha Hospitals, Cambodia, 1 January 2004–15 July 2008

		Number of examinations											
		Any examination			Ziehl-Neelsen			RNA amplification			Culture		
	Children	Negative	Positive	Total	Negative	Positive	Total	Negative	Positive	Total	Negative	Positive	Total
Phase 1													
Clinical cases	21	33	0	33	26	0	26	8	0	8	10	0	10
Confirmed cases*	67	6	93	99	9	84	93	11	66	77	21	60	81
Sub-total	88	39	93	132	35	84	119	19	66	85	31	60	91
Phase 2													
Clinical cases	314	840	0	840	827	0	827	832	0	832	572	0	572
Confirmed cases*	97	84	185	269	154	106	260	94	168	262	132	88	220
Sub-total	411	924	185	1109	981	106	1087	926	168	1094	704	88	792
Phase 3													
Clinical cases	0	0	0	0	0	0	0	0	0	0	0	0	0
Confirmed cases*	96	65	210	275	136	131	267	38	204	242	41	158	199
Sub-total	96	65	210	275	136	131	267	38	204	242	41	158	199
All phases													
Clinical cases	335	873	0	873	853	0	853	840	0	840	582	0	582
Confirmed cases*	260	155	488	643	299	321	620	143	438	581	194	306	500
Total	595	1028	488	1516	1152	321	1473	983	438	1421	776	306	1082

*Cases with laboratory confirmation in at least one result of any type of examination.

two or all of the three methods: microscopy, rRNA amplification and culture (Table 1).

We defined a microbiologically confirmed case ('confirmed case') as a child with at least one positive result by any of the three methods (microscopy, rRNA, culture) and a 'clinical case' as a child without microbiological confirmation of TB.

The emphasis in Phase 1 was on establishing the laboratory methodologies. In Phase 2, samples were obtained from all children hospitalized in Siem Reap with a clinical diagnosis of TB. In Phase 3, specimens from confirmed cases only were collected to enlarge the *M. tuberculosis* isolate collection. The number of children enrolled is thus larger in the second than the first and third Phase (Table 2, Figure 1).

Systematic enrollment of all clinical cases (Phase 2) was restricted to Siem Reap due to logistic difficulties with recruitment in Phnom Penh. This article focuses on this phase.

Table 2 Characteristics of tuberculosis patients during Phase 2, 1 July 2005–31 March 2006, Cambodia

	Clinical cases		Confirmed cases		
	n	Row %	*n*	Row %	Total
Total	314	76.4	97	23.6	411
Hospital					
Siem Reap	314	77.5	91	22.5	405
Phnom Penh	0	0.0	6	100.0	6
Siem Reap only	314	77.5	91	22.5	405
Age group, years					
<1	67	94.4	4	5.6	71
1–<3	59	84.3	11	15.7	70
3–<5	25	75.8	8	24.2	33
5–<10	55	67.9	26	32.1	81
10–<17	108	72.0	42	28.0	150
Sex					
Female	130	72.6	49	27.4	179
Male	183	81.3	42	18.7	225
Missing	1	100.0	0	0.0	1
Disease site					
Respiratory only	134	83.2	27	16.8	161
Non-respiratory only	143	80.3	35	19.7	178
Respiratory and non-respiratory	29	50.0	29	50.0	58
Site not recorded	8	100.0	0	0.0	8

Children with tuberculosis in Phase 2

The main characteristics of all 411 children are summarized in Table 2. During the first month, six

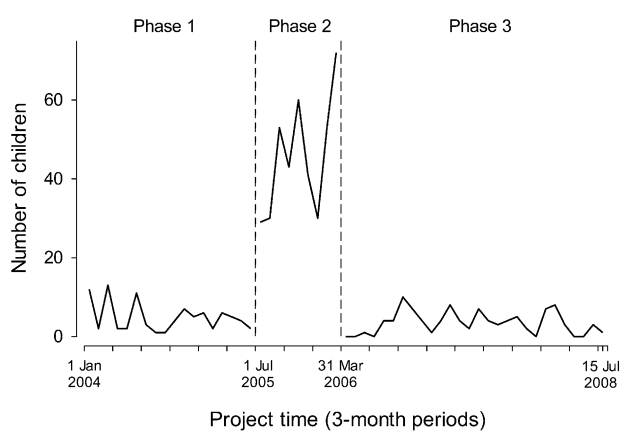

Figure 1 Study phases: Phase 1 encompasses the period from 1 January 2004 to 30 June 2005; Phase 2 encompasses the 9-month period from 1 July 2005 to 31 March 2006; and Phase 3 encompasses the period from 1 April 2006 up to study end on 15 July 2008.

confirmed cases were also reported from Phnom Penh who were excluded from further analysis.

Of the 405 remaining children from Siem Reap, 379 had valid information on admission and laboratory result dates. The result on the first specimen submitted for microscopic and/or rRNA examination was available to the clinician within 48 h following admission for 369 (97.4%) children.

The diagnosis of TB was confirmed in 91 of the 405 children (22.5%, 95% confidence interval [CI] 18.7–26.8). Of these, 83 (91.2%) had a positive rRNA result, 55 (60.4%) had a positive microscopy result, and 47 (51.6%) were positive on both assays. The proportion with confirmed TB was fairly consistent over time after the first 2 months (Figure 2). The mean age among clinical cases was 6.3 years in both sexes. Children with confirmed TB were significantly older than those with a clinical diagnosis (mean age: girls 8.6 and boys 8.7 years). Among all cases, boys predominated (female-to-male ratio 0.80), whereas females predominated slightly (ratio 1.17) in confirmed cases, the opposite being true for clinical cases (ratio 0.71); i.e., 1.65 (1.17/0.71), with more girls than boys across all age groups among the confirmed compared to the non-confirmed cases.

The overall male predominance is explained, at least partially, by the sex imbalance among all admitted children: in a 20-day sample period in Siem Reap in January 2009, the female-to-male ratio among the 1746 admitted children was 0.78. This contrasts with the sex ratio of close to 1 among children aged <15 years in the 2008 national census.[9]

The proportion of children with laboratory confirmation increased with age, and was lowest among the 71 infants (5.6%) and highest among the 81 children aged 5–9 years (32.1%; Figure 3). Of the 231 children aged ⩾5 years, 68 (29.5%, 95%CI 23.9–35.6) had confirmed TB. During this 9-month period, 52 400 children were admitted to the Siem Reap Hospital. The proportion of children with confirmed TB was 1.7, and that of all children with TB 7.7 per 1000 admissions.

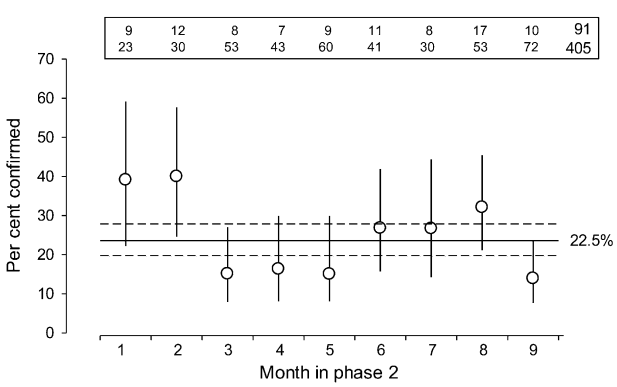

Figure 2 Phase 2, the 9-month period from 1 July 2005 to 31 March 2006, with systematic sampling of all children clinically judged to have tuberculosis, with the proportion of children positive on any laboratory examination. Top row of numbers in box are numerators, bottom number denominators. Circles with vertical lines are monthly point estimates with 95% confidence intervals. The straight horizontal line is the average proportion of cases with laboratory confirmation, the dotted horizontal lines encompass the 95% confidence interval. Siem Reap Hospital, Cambodia.

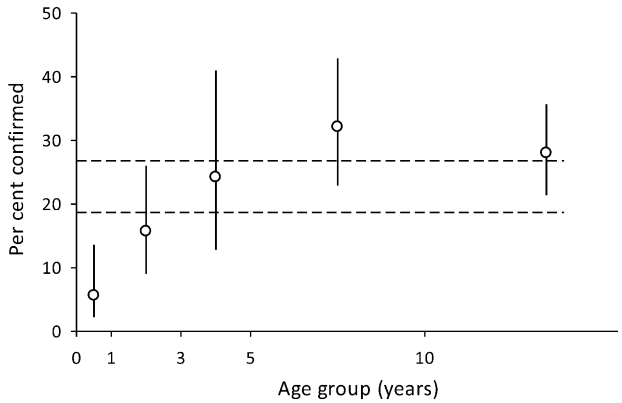

Figure 3 Age-specific proportion of laboratory-confirmed tuberculosis during Phase 2 of systematic clinical sampling. Dashed horizontal lines are 95% confidence intervals around the mean proportion over the entire phase, 1 July 2005–31 March 2006, Siem Reap Hospital, Cambodia.

Microbiological examinations among children in Phase 2

For 303 (74.8%) of the 405 children, at least one specimen was available for culture. Unfortunately, due to inadequate labeling of samples, the specimens of 102 children had to be discarded. Culture failed in one due to contamination. Of the 302 remaining, 221 (73.2%) had neither a positive microscopy nor positive rRNA result in any of the specimens. Three of these 221 (1.4%) had a positive culture result, contributing 7.1% to the total of 42 culture-positive results.

Among the 102 children without a culture result, 7 had a positive rRNA/negative microscopy result, and 2 were positive on both techniques. Of the 91 children with a positive microscopy and/or rRNA result, 8 (8.8%) were positive on microscopy only, 36 (39.6%) on rRNA only, and 47 (51.6%) on both. Thus, 55 (60.4%) had any positive microscopy and 83 (91.2%, 95%CI 85.4–97.0) any positive rRNA result.

In the subset of 42 among the 91 children who also had a positive culture, 1 (2.4%) was positive on microscopy only, 4 (9.5%) on rRNA only, and 34 (81.0%) on both. Thus, 35 (83.3%) had any positive microscopy and 38 (90.5%, 95%CI 81.6–99.4) any positive rRNA result.

The data were further analyzed by specimen rather than children. Specimens examined in routine services independent of the study at the collaborating center in Berne during the same time period were compared with the results obtained in Phase 2 and the entire study period (Figure 4). Within the three groups, i.e., 1) microscopy-positive, rRNA-negative;

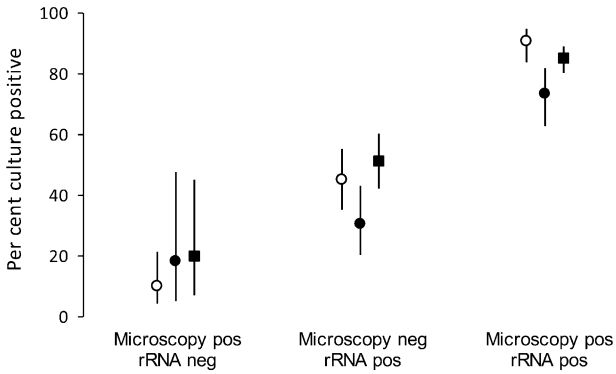

Figure 4 Proportion of culture-confirmed specimens, if the specimen was positive by microscopy and/or the rRNA assay, comparatively at the Institute of Infectious Diseases at the University of Berne (hollow circles) and in Cambodia during Phase 2 (filled circles) and during the entire study period (filled squares).

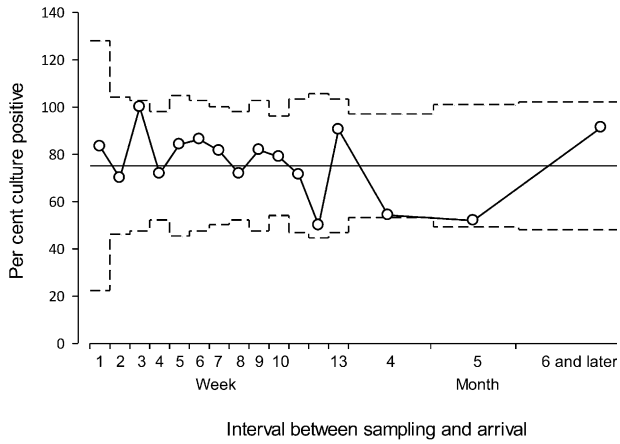

Figure 5 Statistical process control chart of the proportion of specimens positive on microscopy and/or rRNA confirmed by culture, by interval between sampling in Cambodia and arrival at the collaborating laboratory in Switzerland. The line with hollow circles denotes the point proportion of culture positivity at each interval, the straight horizontal line the positive proportion over the entire study period, and the two dashed lines are the upper and lower control limits based on three standard deviations from the mean in each interval.

2) microscopy-negative, rRNA-positive; and 3) both microscopy- and rRNA-positive, the proportion confirmed by culture was lowest in the first, intermediate in the second, and highest in the third group, regardless of setting. In Phase 2 only was there a small difference between Berne (90.7%, 95%CI 83.8–94.9) and Siem Reap (73.4%, 95%CI 62.8–81.9) among those positive on both microscopy and rRNA with culture confirmation.

We finally examined whether transport delay affected viability. Of the 1516 specimens collected during the entire study period, 404 had both a valid culture result and a positive microscopy and/or a positive rRNA result. Of these, 383 had both a known date of sampling in Cambodia and date of specimen arrival in Switzerland. The proportion with a positive culture was determined by shipment delay interval (Figure 5). Of the 383 specimens, 282 (75.2%) were confirmed by culture. To account for batch size variation, we used the statistical process control approach to determine deviations from the expected in each interval.[10] There was no evidence for a negative impact of storage length and the resulting delay between sampling on the proportion of culture positivity.

DISCUSSION

TB diagnosis in children primarily relies on clinical judgment, and laboratory confirmation is frequently unsuccessful.[11] Our operational goals were to enforce collection of appropriate clinical samples in each child with a clinical diagnosis of TB and to provide an efficient laboratory diagnosis without interfering with clinical care.

Specimens were processed and divided into three aliquots for on-site microscopic examination and rRNA detection, and later culture in Switzerland. Implementing an on-site culture facility was deemed inappropriate, considering the costs, complexity, and biosafety. The primary purpose of culture was DST and genotyping.

During the period of systematic clinical sampling, almost one quarter of all cases were microbiologically confirmed. A study from South Africa reported an even higher yield,[12] but any comparison is difficult due to the lack of a gold standard for the clinical diagnosis of childhood TB. Furthermore, the type of health care facility greatly influences the yield.[13] In routine services in Alabama, United States, one quarter of all TB cases in children had bacteriological confirmation.[14] In a nationwide study in the United States, just over 40% of all cases among children and adolescents were confirmed by culture, but comparison is again difficult, as no finer age stratification was provided and our study shows the large variation in yield by age.[15]

We found a large female excess of confirmed compared to non-confirmed cases. This finding is difficult to reconcile. While pediatric TB is frequently reported by sex,[16] we have been unable to identify a report using the same stratification approach differentiating between confirmed and non-confirmed cases by sex. The low yield of laboratory confirmation in infants is likely related to both clinical uncertainty in diagnosis erring on the side of caution, with the increased potential of overdiagnosis, and difficulties in obtaining adequate specimens.

The most reliable and feasible technique for on-site confirmation of clinical diagnosis within 48 h proved to be rRNA amplification, giving a yield of 90% among confirmed cases. This assay has proven highly reliable in other settings, with 94% sensitivity and 100% specificity.[17]

The National TB Program of Cambodia adheres to the standards of the World Health Organization (WHO), requiring notification of all TB patients put on treatment,[18] but stratifying only (direct) sputum smear-positive cases by age,[19] a poor measure of the TB problem among children. In a nationally representative sample of TB case registers in Cambodia,[20] 2.4% of all registered cases were among children, suggesting an extrapolated annual age-specific registration rate of about 20 cases/100 000 children. While hospitalized children inadequately reflect the TB problem in the community at large, close to 1% of children admitted in Siem Reap had TB, suggesting that TB among children remains substantially unrecognized and underreported in Cambodia, as is the case in many other countries,[21] despite recommendations on how to improve the diagnosis of childhood TB in NTPs.[22]

We were unable to equally include all Kantha Bopha hospitals, and our recruitment was thus largely limited to the Siem Reap Hospital. In the latter, however, we were able to systematically and comprehensively recruit children with a clinical diagnosis of TB and to obtain specimens for laboratory examination from virtually all children diagnosed clinically during a 9-month period. This allowed us to determine that, in almost one quarter of children with a clinical diagnosis of TB, an optimized, locally implemented, safe and relatively simple diagnostic service was capable of delivering laboratory confirmation.

CONCLUSION

We demonstrate that implementation of a TB laboratory service in a referral hospital is feasible and that a substantial proportion of TB cases among children, with the exception of infants, can be confirmed rapidly. We utilized rRNA technology with considerable success. Since the conclusion of the study, a simplified approach based on automated DNA extraction and amplification has been successfully tested in the field,[23,24] including among children,[25] and is now promoted by the WHO for rapid scale-up and implementation.[26,27] Rapid progress in development and implementation of molecular techniques can also be expected to assist in the improved diagnosis of TB among children.

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RÉSUMÉ

CONTEXTE : Laboratoire de tuberculose (TB) de l'Hôpital des enfants Jayavarman VII à Siem Reap, un élément des Hôpitaux Kantha Bopha, le complexe d'hôpitaux pédiatriques le plus important du Cambodge.

OBJECTIF : Déterminer l'efficience de l'examen microscopique sur site et de l'amplification de la rRNA chez les enfants où le diagnostic clinique de TB a été porté et chez qui un échantillon a été prélevé pour culture.

RÉSULTATS : Entre le 1er juillet 2005 et le 31 mars 2006, 52 400 enfants ont été hospitalisés ; parmi ceux-ci, 405 étaient atteints de TB, et notamment 91 cas confirmés par le laboratoire (22,5%), soit respectivement 7,7 et 1,7 pour mille admissions. La rRNA a identifié 91,2% des cas confirmés par l'examen microscopique ou le test rRNA. Parmi tous les cas confirmés par la culture, la rRNA en avait identifié 90,5%. La seule culture a contribué à 7,1% de tous les cas confirmés par le laboratoire. Le rendement de la culture à partir d'échantillons conservés n'a pas été affecté par le temps de transport. Chez 97,4% des enfants, la durée maximum avant l'arrivée du résultat au laboratoire sur site a été de 48 h.

CONCLUSION : La mise en œuvre d'un service de mycobactériologie dans un hôpital de référence est réalisable et la technique moléculaire s'avère hautement efficiente. La conservation d'une partie de l'échantillon permet une culture ultérieure sans perte de viabilité liée au temps de transport.

RESUMEN

MARCO DE REFERENCIA: El laboratorio de tuberculosis (TB) en el hospital pediátrico Jayavarman VII en Siem Reap, que forma parte del grupo de hospitales Kantha Bopha, el mayor complejo de hospitales pediátricos de Camboya.

OBJETIVO: Determinar la eficacia de la práctica a nivel local del examen microscópico y la amplificación del ARN ribosomal (ARNr) en las muestras de niños con un diagnóstico clínico de TB y de la recogida de muestras que se remiten para cultivo de micobacterias.

RESULTADOS: Entre el 1° de julio del 2005 y el 31 de marzo del 2006 se hospitalizaron 52 400 niños, de los cuales 405 por TB, y de ellos, 91 casos confirmados en el laboratorio (22,5%), es decir 7,7 y 1,7 casos por 1000 hospitalizaciones, respectivamente. En los casos confirmados por el examen microscópico o por la amplificación del ARNr, 91,2% tuvieron un resultado positivo en la prueba del rRNA y esta prueba detectó 90,5% de todos los casos confirmados por cultivo. El cultivo por sí solo contribuyó al diagnóstico de 7,1% de todos los casos confirmados en el laboratorio. El tiempo de expedición de las muestras conservadas que se remitieron no alteró el rendimiento diagnóstico del cultivo. En 97,4% de los niños el lapso máximo hasta la obtención del resultado del laboratorio local fue 48 h.

CONCLUSIÓN: Es posible implementar el servicio de micobacteriología en un hospital de referencia; la técnica de diagnóstico molecular es de gran eficiencia. El almacenamiento de alícuotas de las muestras permite realizar más tarde los cultivos sin que haya pérdida de la viabilidad por causa del tiempo de expedición.