Laboratory diagnosis of paediatric tuberculosis in the European Union/European Economic Area: analysis of routine laboratory data, 2007 to 2011

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Laboratory confirmation of paediatric tuberculosis (TB) is frequently lacking. We reviewed the range of routine laboratory tests and their performance in different biological samples used to diagnose active TB in children. A questionnaire-based survey was conducted among the European Reference Laboratory Network for TB followed by collection of routine laboratory data on 10,549 paediatric samples tested in 2007 to 2011 at six reference laboratories (in Croatia, Germany, Italy, Latvia, Lithuania and the United Kingdom (UK)). The questionnaire showed that all laboratories used rapid assays. Non-respiratory samples were collected more often in Germany (135/275, 49.1%) and the UK (490/2,140, 22.9%) compared with Croatia (138/2,792, 4.9%), Latvia (222/2,401, 9.2%) and Lithuania (76/1,549, 4.9%). Overall laboratory positivity rates (isolation of *Mycobacterium tuberculosis* complex and/or identification of its nucleic acids in a sample) were higher in lymph node and gastric aspirate samples (14/203 (6.9%) and 43/1,231 (3.5%)) than in sputum samples (89/4,684 (1.9%)). Pooled sensitivity, specificity, positive and negative predictive values and accuracy of molecular assays assessed against solid or liquid culture were 79.2%, 93.6%, 67.1%, 96.5% and 91.6%, respectively. A more intensive approach in obtaining gastric aspirate and non-respiratory samples may increase laboratory confirmation of paediatric TB. Major effort is needed in optimisation and validation of molecular tests in these samples.

Introduction

Tuberculosis (TB) affects globally about 490,000 children under 15 years-old, with 64,000 related deaths occurring every year [1]. Paediatric TB is an indicator of recent transmission in the population. Often, children experience more severe forms of the disease, such as miliary or meningeal TB [2,3]. Young children are rarely able to expectorate sputum; therefore, other respiratory samples, such as gastric aspirates (GA) or bronchoalveolar lavages (BAL), can be obtained for diagnostic purposes, although these procedures are more unpleasant for a child than sputum collection [1,3-5]. Collection of non-respiratory samples (lymph node (LN), pus or tissue biopsy) is necessary to diagnose extrapulmonary TB; however, these procedures are relatively invasive [6,7].

TB in children often has a paucibacillar nature, resulting in microscopy smears that are negative [3,4,8]. Culture isolation, a more sensitive method, takes up to 14 days due to slow growth of *Mycobacterium tuberculosis* complex (MTB(C)) bacteria; however, even culture is seldom positive in paediatric specimens due to very few bacilli present in a sample [3,4,8]. Further full drug susceptibility testing (DST) can only be done once an isolate is available and takes two more weeks [9,10].

Consequently, the diagnosis of paediatric TB often relies on a combination of clinical judgment and radiological findings, prompting initiation of treatment without clear laboratory evidence [1,8,9,11,12]. Unlike laboratory criteria, there is as yet no universally applied diagnostic algorithm based on clinical and radiological criteria that are objective [8,11,12].

The most important advance in TB diagnosis is the introduction of the internationally endorsed molecular assay Xpert MTB/RIF (Cepheid Inc., Sunnyvale, CA, United States), which identifies MTB and rifampicin resistance from sputum samples [13]. A growing body of evidence supports the adequacy of Xpert MTB/RIF for adult TB diagnosis [14-16].

Recent studies have investigated the performance of the Xpert MTB/RIF system in children and showed promising results when the test is applied to sputum, nasopharyngeal aspirates (NPA), GA or non-respiratory samples [4,6,7,17-19]. However, there are limited data on the usefulness of molecular assays for paediatric TB diagnosis in Europe, where TB prevalence is low [20].

The aim of the study was to give an overview of the range of routine diagnostic tests and their performance for different types of samples used to diagnose active TB in children across European laboratories.

Methods

Study design
The study was planned in two stages. Firstly, we invited all (38) national TB reference laboratories across the European Union (EU)/European Economic Area (EEA) countries that are members of the European Reference Laboratory Network for TB (ERLN-TB) – an initiative supported by the European Centre for Disease Prevention and Control (ECDC) [21] – to participate in a questionnaire-based survey. The questionnaire aimed to determine the variety of algorithms used to diagnose paediatric TB and the type of samples collected from children; it contained 53 questions and was based on an earlier questionnaire used across the ERLN-TB [22].

Secondly, six laboratories of the network (referred to hereafter as study sites) agreed to provide their routine data for all consecutive primary samples and reference cultures referred for diagnosis from children younger than 15 years-old with suspected TB during 2007 to 2011.

Primary samples were defined as specimens referred to a reference laboratory for primary diagnostics, while reference cultures were cultures isolated by local laboratories and referred to a reference laboratory for confirmation, DST or molecular typing.

Low-incidence western and central European settings were represented by the following laboratories: the National Reference Centre for Mycobacteria at Forschungszentrum Borstel, Borstel, Germany; San Raffaele Scientific Institute, in collaboration with the Institute 'Villa Marelli', Niguarda Caˊ Granda Hospital, Milan, Italy; the National Mycobacterium Reference Laboratory, Public Health England, London, United Kingdom (UK) and the National Mycobacterium Reference Laboratory, National Institute of Public Health, Zagreb, Croatia (TB incidence in 2012 being

5.6, 6.7, 15.0 and 14.0 cases per 100,000 population in the respective countries [23]).

The National TB Reference Laboratory at the Latvian Infectology Centre, Upeslejas, Latvia, and the Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital, Vilnius, Lithuania, represented medium-incidence eastern European countries, with TB incidence in 2012 being 53.0 and 66.0 cases per 100,000 population, respectively [23].

Only partial data were available from the Italian site for 2011; this is the only site that does not have a national reference laboratory function, covering mainly the Lombardy region of Italy. Germany provided data for 2011 only. We did not exclude control samples, as there were only a few of those.

The laboratory data were collected using an Excel-based tool, with line-listing by sample. The Croatian, German, Italian and UK sites received a small proportion of all paediatric samples in the country (estimated by the laboratory directors as less than 10%), while the Lithuanian site covered approximately half and the Latvian site all paediatric samples in their countries.

Laboratory positivity was confirmed by a positive culture (on solid or liquid media) identified as MTB(C) or non-tuberculous mycobacteria (NTM) and/or by the identification of MTB(C) or NTM nucleic acid directly in a sample by a molecular assay.

Ethics statement
Routine laboratory data were sent to the Robert Koch Institute, Berlin, Germany, without personal identifiers. The study obtained a waiver of informed consent and ethics review permission from the Robert Koch Institute.

Data analysis
Per-sample and per-patient analyses were conducted. Samples were categorised as 'respiratory' (sputum, BAL, GA, pleural fluid and other respiratory samples such as NPA), 'non-respiratory' (cerebrospinal fluid (CSF), LN, pus, blood, other tissue biopsy, urine and other non-respiratory samples such as pericardial fluid) and 'unknown' (where exact information on sample type was missing from the records).

Calculations related to the MTB(C) did not include the *Mycobacterium bovis* strain used in the Bacillus Calmette–Guérin (BCG) vaccine.

The incremental positivity rate, defined as additional sensitivity gained by testing more than one sample from the same patient, was calculated for GA samples.

Associations between correct diagnostic yield and sample type were tested using Pearson's chi-squared test or Fisher's exact test. Statistical tests were two-sided at alpha=0.05.

Laboratory diagnosis of paediatric tuberculosis: types of paediatric primary samples received across the six study sites, 2007–2011 (n=9,157)

Site	Total number of samples received	Sample type		
		Respiratory[a] n (%)	Non-respiratory[a] n (%)	Unknown n (%)
Croatia CIPH	2,792	2,650 (94.9)	138 (4.9)	4 (0.1)
Germany[b] NRCM	275	129 (46.9)	135 (49.1)	11 (4.0)
Italy[b] HSR	340	NA	NA	NA
Latvia NTRL	2,401	2,167 (90.3)	222 (9.2)	12 (0.5)
Lithuania TBL	1,549	1,464 (94.5)	76 (4.9)	9 (0.6)
United Kingdom NMRL	2,140	1,643 (76.8)	490 (22.9)	7 (0.3)
All sites	**9,157[c]**	**8,053 (87.9)**	**1,061 (11.6)**	**43 (0.5)**

CIPH: Croatian National Institute of Public Health, National Mycobacterium Reference Laboratory; HSR: San Raffaele Scientific Institute in collaboration with the Institute 'Villa Marelli', Niguarda Ca´ Granda Hospital; NA: data not available; NMRL: National Mycobacterium Reference Laboratory; NRCM: National Reference Centre for Mycobacteria at Forschungszentrum Borstel; NTRL: National TB Reference Laboratory at the Latvian Infectology Centre; TBL: Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital.

[a] Respiratory samples: sputum, gastric aspirate, bronchoalveloar lavage, pleural fluid, other respiratory samples (e.g. nasopharyngeal aspirate). Non-respiratory samples: cerebrospinal fluid, lymph node, pus, other biopsy tissue, urine, other non-respiratory samples (e.g. pericardial fluid).

[b] Data from 2011 only.

[c] Does not include the 340 samples from Italy.

The results of the different types of molecular tests were pooled since the number of samples did not allow stratified analysis of each test. Sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) and the accuracy of the pooled molecular assays was assessed against culture and DST results in samples tested by both methods.

The answers from the survey questionnaire were entered into EpiData (EpiData Association, Odense, Denmark) and analysed using STATA (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP). The routine laboratory data were collected from the study sites using a Microsoft Excel spreadsheet and analysed in STATA.

Results

Questionnaire-based survey

A total of 21 TB reference laboratories from Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Estonia, Finland, Germany, Greece, Hungary, Italy (two laboratories), Latvia, Lithuania, Norway, Portugal, Slovenia, Spain (two laboratories), Sweden and the UK sent their responses.

The population served by these laboratories varied between 400,000 and 20,000,000 inhabitants. Annually, the laboratories receive a median of 70 (range: 6–950) paediatric primary samples and reference cultures representing a median of 3.1% (range: 0.3–8.3%) of the total laboratory workload.

Of the 21 laboratories, 16 receive primary samples; the Latvian and Lithuanian laboratories receive primary samples only. Five laboratories receive reference cultures only (Belgium, Czech Republic, Finland, Norway and Spain). The most common sample type received from children is sputum (16 laboratories), followed by GA and BAL (15 laboratories each). A total of 11 laboratories reported receiving more than one type of sample from each child.

All laboratories receiving primary samples perform smear microscopy, molecular identification, culture (solid or liquid media, commercial or in-house) and first- and second-line DST. All undergo international quality assurance, demonstrating satisfactory performance.

The most frequently used direct rapid molecular identification assay included Xpert MTB/RIF (n=10) and GenoType MTBDR*plus* (Hain Lifescience, GmbH, Germany) (n=8); 10 laboratories used more than one assay. All laboratories rapidly identified MTB(C) from positive culture using GenoType CM/AS (Hain Lifescience, GmbH, Germany) (n=11), BD MGIT TBc Id (Becton Dickinson, United States) and GenoType MTBDR*plus* assays (n=8 each). Nine laboratories

Laboratory diagnosis of paediatric tuberculosis: type and percentage of paediatric primary samples received across five study sites, 2007–2011 (n=9,157)
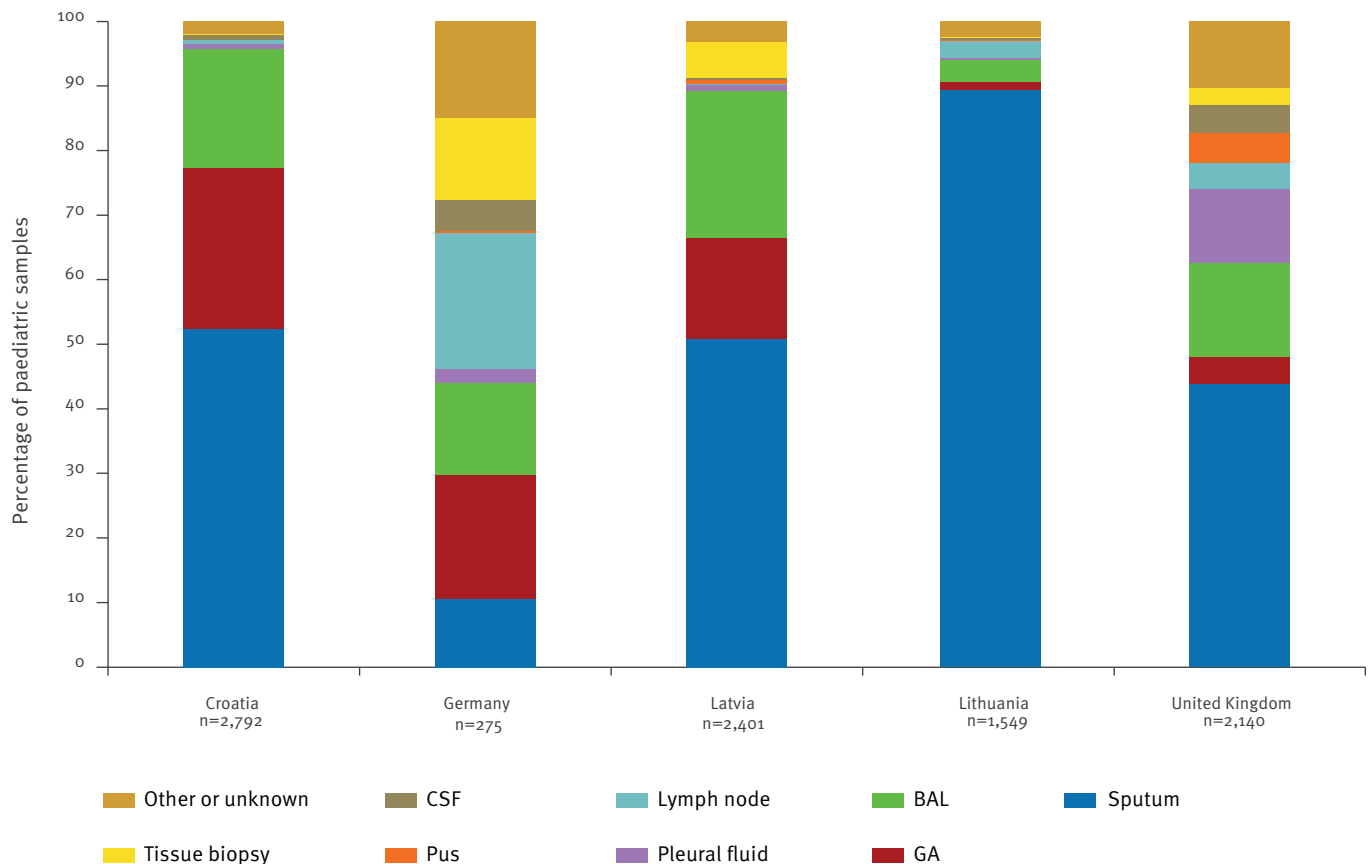
CSF: cerebrospinal fluid; BAL: bronchoalveolar lavage; GA: gastric aspirate.

Data from the Italian laboratory were not included, since available data specified only that the samples were either respiratory or non-respiratory, without further information on specific sample type.

routinely used GenoType MTBDRsl (Hain Lifescience, Germany) for rapid second-line DST.

In 17 laboratories, the algorithms used to diagnose paediatric and adult TB did not differ; four laboratories reported the use of extra tests for children. In Latvia and Lithuania, for example, when there was a positive culture from a biopsy sample from a child GenoType MTBC (Hain Lifescience, Germany) was used to distinguish between MTB and *M. bovis* BCG.

Routine laboratory data

Number and range of primary samples received
A total of 9,157 primary samples and 1,392 reference cultures were received from children across all six study sites within the five-year study period (Table 1).

Of all primary samples, the vast majority (8,053/9,157, 87.9%) were respiratory, including 4,974 sputum (61.8%), 1,467 BAL (18.2%), 1,232 GA (15.3%) and 298 pleural fluid (3.7%) samples; 1,061 samples were

non-respiratory, including 230 tissue biopsy samples other than LN (21.7%), 205 LN (19.3%), 182 urine (17.2%), 140 CSF (13.2%) and 118 pus (11.1%) (Figure).

In the Croatian, Latvian and Lithuanian sites, less than 10% of all paediatric samples were non-respiratory (138/2,792, 4.9%; 222/2,401, 9.2%; 76/1,549, 4.9%, respectively), whereas in the UK site, this proportion was higher, 490/2,140 (22.9%). The German site received more non-respiratory than respiratory primary samples (135/275, 49.1% and 129/275, 46.9%, respectively), with LN being the most common sample type (58/275, 21.1%) (Table 1, Figure).

Microscopy, culture and drug susceptibility testing
Most of the primary samples (8,176/9,157, 89.3%) were subjected to smear microscopy, resulting in a 3.3% (268/8,176) positivity rate (Table 2). More non-respiratory than respiratory samples were positive by microscopy (82/816, 10.0% vs 181/7,320, 2.5%, p<0.0001).

Laboratory diagnosis of paediatric tuberculosis: tests performed on paediatric primary samples across the six study sites, 2007–2011 (n=9,157)

Type of laboratory test	Result	Sample type		All samples[b]
		Respiratory[a] n (%)	Non-respiratory[a] n (%)	n (%)
Smear microscopy	All	7,320 (100)	816 (100)	8,176 (100)
	Positive	181 (2.5)	82 (10.0)	268 (3.3)
	Negative	7,139 (97.5)	734 (90.0)	7,908 (96.7)
Culture[c]	All	7,749 (100)	1,015 (100)	8,806 (100)
	Positive[d]	392 (5.1)	142 (14.0)	541 (6.1)
	Positive: MTB(C)[e]	149 (1.9)	40 (3.9)	195 (2.2)
	Positive: NTM	211 (2.7)	89 (8.8)	314 (3.6)
	Negative	7,213 (93.1)	848 (83.5)	8,096 (91.9)
	Contaminated	144 (1.8)	25 (2.5)	169 (1.9)
Direct rapid molecular identification of MTB(C) in primary samples[f]	All	308 (100)	258 (100)	578 (100)
	Positive for MTB(C)[e]	64 (20.8)	28 (10.8)	93 (16.1)
	Negative	242 (78.6)	218 (84.5)	471 (81.5)
	Indeterminate	2 (0.6)	12 (4.7)	14 (2.4)
Direct rapid molecular DST on primary samples	All	64 (100)	28 (100)	93 (100)
	Rifampicin susceptible	59 (92.2)	26 (92.9)	85 (91.4)
	Rifampicin resistant	4 (6.2)	2 (7.1)	7 (7.5)
	Indeterminate	1 (1.6)	0 (0.0)	1 (1.1)

BCG: Bacillus Calmette–Guérin; DST: drug susceptibility testing; MTB(C): *Mycobacterium tuberculosis* complex; NTM: non-tuberculous mycobacteria.

[a] Respiratory samples: sputum, gastric aspirate, bronchoalveloar lavage, pleural fluid, other respiratory samples (e.g. nasopharyngeal aspirate); non-respiratory samples: cerebrospinal fluid, lymph node, pus, other biopsy tissue, urine, other non-respiratory samples (e.g. pericardial fluid).

[b] Including samples of unknown type.

[c] Either Löwenstein–Jensen, Mycobacteria Growth Indicator Tube or other solid or liquid media. Only one positive culture result per sample was analysed.

[d] 32 of the 541 positive culture samples were either not further identified or were identified as *M. bovis* BCG.

[e] *M. bovis* BCG not included.

[f] Used the following assays: Croatia: GeneXpert MTB/RIF, Amplified MTB Direct Test GenProbe; Germany: GeneXpert MTB/RIF, GenoType MTBDR*plus*, in-house test; Italy: GeneXpert MTB/RIF, INNO-LiPA; Latvia: GeneXpert MTB/RIF; Lithuania: GeneXpert MTB/RIF; United Kingdom: GeneXpert MTB/RIF, GenoType MTBDR*plus*, INNO-LiPA, IS6110 sequencing.

Almost all (8,806/9,157, 96.2%) samples were cultured and a positive culture was obtained in 541 (6.1%) of the 8,806 primary samples. Culture was more often positive from non-respiratory than from respiratory samples (142/1,015, 14.0% vs 392/7,749, 5.1%, p<0.0001 for all positive cultures; 40/1,015, 3.9% vs 149/7,749, 1.9%, p=0.0001 for MTB(C)-positive cultures) (Table 2).

Across all sites, the culture contamination rate (on solid and liquid media) was 169/8,806 (1.9%) (Table 2), ranging from 0/2,884 (0%) at the Croatian to 16/240 (6.7%) at the German site.

Per-sample analysis showed that MTB(C) was detected in 236/8,851 (2.7%) of primary samples with the highest rates at the German and UK sites, 23/275 (8.4%) and 107/2,132 (5.0%), respectively (Table 3). The sample positivity rates for MTB(C) were significantly higher in pus (p=0.0005), LN (p<0.0001), CSF (p=0.002) and other tissue biopsy (p=0.002) samples compared with sputum samples. Among respiratory samples, MTB(C) was more commonly detected in GA samples than in sputum samples (43/1,231, 3.5% vs 89/4,684, 1.9%, p=0.002). No statistically significant difference between MTB(C) detection rates in GA or BAL samples was observed. Per-patient analysis showed that MTB(C) was isolated in 156/5,156 (3.0%) of children (Table 4). MTB(C) was detected in 31/643 (4.8%) patients who submitted GA samples; of them, 21/31 patients submitted at least two GA samples. The first GA sample identified 16 positive patients, the second identified an additional four and the third, another positive patient.

Multidrug resistance (MDR), defined as resistance to at least rifampicin and isoniazid, was seen in 10/156 (6.4%) of patients ranging from 0/23 and 0/4 at Croatian

Laboratory diagnosis of paediatric tuberculosis: paediatric primary samples identified as MTB(C) across five study sites, 2007–2011 (n=236)

Site	Number of samples identified as MTB(C)[a]/total number per sample type (%)								
	Sputum	GA	BAL	Lymph node	Pus	CSF	Tissue biopsy	Other samples	**All samples**
Croatia CIPH	6/1,402 (0.4)	20/694 (2.9)	9/516 (1.7)	1/20 (5.0)	0/0 (0.0)	0/19 (0.0)	0/3 (0.0)	1/138 (0.7)	**37/2,792 (1.3)**
Germany[b] NRCM	1/29 (3.4)	9/53 (17.0)	5/39 (12.8)	2/58 (3.4)	0/1 (0.0)	1/13 (7.7)	2/35 (5.7)	3/47 (6.4)	**46/2,397 (1.9)**
Latvia NTRL	16/1,219 (1.3)	4/378 (1.1)	13/546 (2.4)	1/2 (50.0)	1/10 (10.0)	1/9 (11.1)	6/135 (4.4)	4/98 (4.1)	**107/2,132 (5.0)**
Lithuania TBL	13/1,099 (1.2)	0/17 (0.0)	2/54 (3.7)	4/40 (10.0)	0/1 (0.0)	0/6 (0.0)	1/1 (100)	3/37 (8.1)	**23/1,255 (1.8)**
United Kingdom NMRL	53/935 (5.7)	10/89 (11.2)	7/312 (2.2)	6/83 (7.2)	8/102 (7.8)	7/91 (7.7)	6/56 (10.7)	10/464 (2.2)	**107/2,132 (5.0)**
All sites	**89/4,684 (1.9)**	**43/1,231 (3.5)**	**36/1,467 (2.5)**	**14/203 (6.9)**	**9/114 (7.9)**	**9/138 (6.5)**	**15/230 (6.5)**	**21/784 (2.7)**	**236/8,851 (2.7)**

BAL: bronchoalveolar lavage; BCG: Bacillus Calmette–Guérin; CIPH: Croatian National Institute of Public Health; National Mycobacterium Reference Laboratory; CSF: cerebrospinal fluid; GA: gastric aspirate; MTB(C): *Mycobacterium tuberculosis* complex; NMRL: National Mycobacterium Reference Laboratory; NRCM: National Reference Centre for Mycobacteria at Forschungszentrum Borstel; NTRL: National TB Reference Laboratory at the Latvian Infectology Centre; TBL: Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital.

[a] By isolation of MTB(C) culture or identification of MTB(C) nucleic acid in a sample. *M. bovis* BCG results are not included.
[b] Data from 2011 only.

Laboratory-confirmed paediatric cases[a] across the six study sites (with primary samples only), 2007–2011 (n=156)

Site	Type of primary sample		Identification of MTB(C)	Drug susceptibility testing
	Sputum	Gastric aspirate	n MTB(C)-positive patients[c]/N patients (%)	n with MDR/ N MTB(C)-positive patients[c] (%)
	n samples/ N patients (ratio)	n samples/ N patients (ratio)		
Croatia CIPH	1,402/1,006 (1.4)	694/391 (1.8)	23/1,681 (1.4)	0/23 (0.0)
Germany[b] NRCM	29/21 (1.4)	53/22 (2.4)	7/152 (4.6)	1/7 (14.3)
Italy[b] HSR	NA	NA	4/200 (2.0)	0/4 (0.0)
Latvia NTRL	1,219/504 (2.4)	378/170 (2.2)	40/1,151 (3.5)	3/40 (7.5)
Lithuania TBL	1,385/774 (1.8)	18/13 (1.4)	17/886 (1.9)	3/17 (17.7)
United Kingdom NMRL	939/382 (2.5)	89/47 (1.9)	65/1,086 (6.0)	3/65 (4.6)
All sites	**4,974/2,687 (1.9)**	**1,232/643 (1.9)**	**156/5,156 (3.0)**	**10/156 (6.4)**

CIPH: Croatian National Institute of Public Health, National Mycobacterium Reference Laboratory; HSR: San Raffaele Scientific Institute in collaboration with the Institute 'Villa Marelli', NiguardaCa´ Granda Hospital;MDR: multidrug resistance (resistant to rifampicin and isoniazid); NA: data not available; NMRL: National Mycobacterium Reference Laboratory; MTB(C): *Mycobacterium tuberculosis* complex; NRCM: National Reference Centre for Mycobacteria at Forschungszentrum Borstel; NTRL: National TB Reference Laboratory at the Latvian Infectology Centre; TBL: Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital.

[a] By isolation of MTB(C) culture or identification of MTB(C) nucleic acid from any of the patient´s samples.
[b] Data from 2011 only.
[c] *M. bovis* BCG not included.

Laboratory diagnosis of paediatric tuberculosis: performance of molecular assays compared with culture in detecting MTB(C) in primary samples across the six study sites[a], 2007–2011

Sample type and smear results	Sensitivity[b]		Specificity[c]		Positive predictive value[d]		Negative predictive value[e]		Accuracy[f]	
	n/N	% (95% CI)	n/N	% (95% CI)	n/N	% (95% CI)	n/N	% (95% CI)	n/N	% (95% CI)
All[g,h]	57/72	79.2 (68.0–87.8)	411/439	93.6 (90.9–95.7)	57/85	67.1 (56.0–76.9)	411/426	96.5 (94.3–98.0)	468/511	91.6 (88.8–93.8)
All smear positives	42/46	91.3 (79.2–97.6)	41/56	73.2 (59.7–84.2)	42/67	62.7 (60.3–84.5)	41/45	91.1 (78.8–97.5)	83/102	81.4 (72.4–88.4)
All smear negatives	11/21	52.4 (29.8–74.3)	287/299	96.0 (93.1–97.9)	11/23	47.8 (26.8–69.4)	287/297	96.6 (93.9–98.4)	298/320	93.1 (89.8–95.6)
Respiratory[g]	48/54	88.9 (77.4–95.8)	224/236	94.9 (91.3–97.3)	48/60	80.0 (67.7–89.2)	224/230	97.4 (94.4–99.0)	272/290	93.8 (90.4–96.3)
Respiratory smear positives	40/42	95.2 (83.8–99.4)	15/20	75.0 (50.9–91.3)	40/45	88.9 (75.9–96.3)	15/17	88.2 (63.6–98.5)	55/62	88.7 (78.1–95.4)
Respiratory smear negatives	5/8	62.5 (24.5–91.5)	161/168	95.8 (91.6–98.3)	5/12	41.7 (15.2–72.3)	161/164	98.2 (94.7–99.6)	166/176	94.3 (89.8–97.2)
Non-respiratory[g]	7/16	43.8 (19.8–70.1)	177/192	92.2 (87.4–95.6)	7/22	31.8 (13.9–54.9)	177/186	95.2 (91.0–97.8)	184/208	88.5 (83.3–92.5)
Non-respiratory smear positives	2/4	50.0 (6.8–93.2)	24/34	70.6 (52.5–84.9)	2/12	16.7 (2.1–48.4)	24/26	92.3 (74.9–99.1)	26/38	68.4 (51.3–82.5)
Non-respiratory smear negatives	5/12	41.7 (15.2–72.3)	118/123	95.9 (90.8–98.7)	5/10	50.0 (18.7–81.3)	118/125	94.4 (88.8–97.7)	123/135	91.1 (85.0–95.3)
Sputum[g]	34/37	91.9 (78.1–98.3)	54/57	94.7 (85.4–98.9	34/37	91.9 (78.1–98.3)	54/57	94.7 (85.4–98.9	88/94	93.6 (86.6–97.6
Sputum smear positives	32/33	97.0 (84.2–99.9)	6/9	66.7 (29.9–92.5)	32/35	91.4 (76.9–98.2)	6/7	85.7 (42.1–99.6	38/42	90.5 (77.3–97.3
Sputum smear negatives	1/3	33.3 (0.8–90.6)	48/48	100 (92.6–100)	1/1	100 (2.5–100)	48/50	96.0 (86.3–99.5)	49/51	96.0 (86.5–99.5)
Gastric aspirates[g]	8/10	80.0 (44.4–97.5)	95/99	96.0 (90.0–98.9)	8/12	66.7 (34.9–90.1)	95/97	97.9 (92.7–99.7)	103/109	94.5 (88.4–97.9)
Bronchoalveolar lavage[g]	6/7	85.7 (42.1- 99.6)	55/59	93.2 (83.5- 98.1)	6/10	60.0 (26.2–87.8)	55/56	98.2 (90.4–100)	61/66	92.4 (83.2–97.5)

CI: confidence interval; MTB(C): *Mycobacterium tuberculosis* complex.

a   CIPH: Croatian National Institute of Public Health, National Mycobacterium Reference Laboratory; HSR: San Raffaele Scientific Institute in collaboration with the Institute 'Villa Marelli', NiguardaCa´ Granda Hospital; NMRL: National Mycobacterium Reference Laboratory; NRCM: National Reference Centre for Mycobacteria at Forschungszentrum Borstel; NTRL: National TB Reference Laboratory at the Latvian Infectology Centre; TBL: Tuberculosis Bacteriology Laboratory at the Infectious Diseases and Tuberculosis Hospital.
b   Number of true positives/number of true positives + number of false negatives.
c   Number of true negatives/number of true negatives + number of false positives.
d   Number of true positives/number of true positives + number of false positives.
e   Number of true negatives/number of true negatives + number of false negatives.
f   Number of true positives + number of true negatives/total number of tested samples.
g   Number of smear negatives + number of smear positives + number with smear result unknown.
h   Number of respiratory samples + number of non-respiratory samples + number of samples of unknown type.

and Italian sites to 3/17 of cases at the Lithuanian site (Table 4).

Rapid molecular test performance for *Mycobacterium tuberculosis* complex and drug-resistance detection

Routine use of NAAT was initiated in 1996 at the Croatian, in 1994 at the German and Italian, in 2010 at the Latvian, in 2008 at the Lithuanian and in 1999 at the UK sites. A variety of commercial and in-house assays were used on 578/9,157 (6.3%) of samples.

The bacteria in respiratory samples were more often directly identified as MTB(C) than those in non-respiratory samples (64/308, 20.8% vs 28/258, 10.9%, p=0.001). The rate of indeterminate results was higher among non-respiratory samples (12/258, 4.7% vs 2/308, 0.6%, p=0.0024) (Table 2).

A total of 511 primary samples were tested by both molecular assay and culture for MTB(C) identification. Compared with liquid or solid culture, a rapid molecular test – based on the pooled data analysis – had an overall (for all samples types) sensitivity of

57/72 (79.2%), specificity of 411/439 (93.6%), PPV of 57/85 (67.1%), NPV of 411/426 (96.5%) and accuracy of 468/511 (91.6%) for MTB(C) detection (Table 5). The sensitivity of molecular tests for smear-positive samples vs smear-negative samples was significantly higher (42/46, 91.3% vs. 11/21, 52.4%, p=0.0006), while they were less specific (41/56, 73.2% vs 287/299, 96.0%, p=0.0001). The lower specificity in the smear-positive samples was due to 12 false-positive results at the UK site tested by INNO-LiPA Rif.TB (Innogenetics, Ghent, Belgium) and Xpert MTB/RIF, two at the German site tested by Xpert MTB/RIF and one at Croatian site tested by Xpert MTB/RIF.

The molecular tests detected MTB(C) in 48/54 (88.9%) of respiratory and 7/16 (43.8%) of non-respiratory samples (p=0.0004); the specificity was 224/236 (94.9%) and 177/192 (92.2%), respectively. For both respiratory and non-respiratory samples, the sensitivity was again higher for smear-positive than for smear-negative samples; however, the specificity was higher for smear-negative samples for both groups of samples (Table 5).

Molecular assays produced 15/511 (2.9%) of false-negative results and 28/511 (5.5%) of false-positive results for MTB(C) identification; discordant results were related to all types of samples. Mutations coding resistance to rifampicin were detected in 7/93 (7.5%) of all primary samples tested by rapid assays (Table 2). Molecular and phenotypic assays were fully concordant with DST.

Non-tuberculous mycobacteria and *Mycobacterium bovis* BCG

Approximately half (989/1,903, 52.0%) of all positive isolates (grown from primary samples and those received as reference cultures) were identified as NTMs at the Croatian (43/94), German (54/90), Italian (26/50) and UK (862/1,564, 55.1%) sites. NTMs were less common at the Latvian (1/67) and Lithuanian (3/38) sites. A proportion of all positive isolates (224/1,903, 11.8%) were identified as *M. bovis* BCG.

NTMs were isolated at the highest rate from LN samples (27/203, 13.3%). NTMs were seen in 136/4,958 (2.7%) of all paediatric patients, with *M. abscessus* as the most frequently identified.

Discussion

This large study reviewed the analysis of over 10,000 samples tested across a number of European TB reference laboratories and showed the availability and use of all conventional and modern diagnostic techniques across these settings. Although the diagnostic algorithms were similar across laboratories, approaches towards collecting paediatric non-respiratory samples differ between European sites, with very few samples other than sputum obtained from children in Latvia and Lithuania.

Because young children can rarely expectorate but instead swallow their sputum, aspiration of the gastric content is the best (and the least unpleasant) procedure for obtaining a sputum sample suitable for further diagnostics [4,5,19,24]. Our study showed that, compared with sputum samples, testing GA more often results in positive findings. Recent data by Fiebig et al. support this evidence [25]: they report widespread use of GA in Germany and a high (63%) yield of culture and NAAT in paediatric specimens, confirming that it is possible to reliably diagnose TB in children and disagreeing with the common notion of 'difficulties' in laboratory confirmation of paediatric disease.

Our data suggest that collection of several GA samples might help identify further cases, in line with previously published data [5]. Larger studies are needed to assess the differences in diagnostic yield in serial samples.

Extrapulmonary samples also tested positive more frequently than sputum samples did, placing emphasis on the usefulness of collecting non-respiratory materials, in line with some previously published results that showed a good performance of fine-needle aspiration biopsy of LN in confirming TB [26]. Considering the observed small proportion of non-respiratory samples received at the Latvian and Lithuanian sites, it would seem reasonable to expect a low incidence of extrapulmonary TB there. However, crude comparison with World Health Organization (WHO) surveillance data for 2011 showed that while in Croatia, Germany, Italy and the UK extrapulmonary TB represented the minority of paediatric cases (15%, 17%, 19% and 37%, respectively), the majority of notified cases in Latvia and Lithuania were, in contrast, extrapulmonary (59% and 92%, respectively) [23], pointing towards predominantly non-laboratory based diagnosing. Within the framework of our study, infrequent collection of samples other than sputum might be explained by differences in the referral population, with a large number of asymptomatic children being screened for TB as part of contact tracing in medium-incidence settings, such as those of the participating Baltic countries [23]. Relatively low MTB(C)-positivity rates at the Latvian and Lithuanian sites also reflect this screening strategy. There, a lower level of clinical suspicion might be the reason for not carrying out more invasive procedures. Another explanation might be a more cautious attitude of eastern European physicians in Latvia and Lithuania in collecting GA or non-respiratory samples from children, related to the higher invasiveness of these procedures compared with sputum collection, and lack of confidence in the success of laboratory diagnosis. The rates of notified extrapulmonary TB across the EU/EEA vary between 4% and 48% (37.7% in children during 2002 to 2011), possibly reflecting the challenges and differences in diagnosis and confirming the urgent need for improvement [27,28].

Analysis of the performance of the pooled molecular tests showed that the sensitivity was comparable to that reported for the Xpert MTB/RIF in children in other studies: 79.5% for sputum and GA in Vietnam, 90.0 and 68.8% for sputum and GA samples in Zambia, 73.6% and 75.9% in South Africa using NPA and sputum samples, 54.7% in Tanzania [4,6,17-19]. The molecular tests were more sensitive in detecting MTB(C) in respiratory samples than in non-respiratory samples as previously described [17,29] which may be explained by the design of the molecular tests originally aimed to work in respiratory samples. We observed no differences in sensitivity and specificity for sputum, GA, or BAL samples, as previously observed [19].

The specificity was lower in smear-positive samples than in smear-negative samples. It might be an artefact resulting from the pooling of different molecular methods or the low specificity of molecular tests for the DNA target or the use of an imperfect gold standard method. Although culture is the recognised current gold standard for TB diagnosis, it still might be an imperfect comparator, possibly leading to failure to diagnose some cases [17].

Similar performance characteristics of INNO-LiPA Rif. TB and Xpert MTB/RIF used for the diagnosis of TB in adults were reported in Italy and the UK [7,29]; however, these results were primarily evaluating a single rapid method (Xpert MTB/RIF) on a single type of sample. High incidence of TB may explain the higher PPV observed in studies conducted outside Europe (100% for sputum and GA in Vietnam, 81.8% and 86.8% for sputum and GA samples respectively in Zambia, 92.8% and 89.7% in South Africa using NPA and sputum samples) [6,17-19], compared with our study.

Our study showed that NTMs were more frequently isolated from children than MTB(C). *M. bovis* BCG was frequently isolated in Latvia and Lithuania where BCG vaccination is a national policy. Our data showed that *M. abscessus* was the predominant NTM isolated from children while previously was the *M. avium* complex [30].

This study has several limitations. The analysis was based on subnational data, leading to possible selection bias and restriction of the geographical representativeness of the study. A diagnostic bias resulting from more invasive samples being taken when there is a higher level of clinical suspicion may cause the higher positivity rates in non-respiratory samples. Two sites could provide 2011 data only. Small case numbers forced 'pooling' of the NAAT results and our approach ignores the possible heterogeneity of results or differences in ROC curve of various tests. The retrospective laboratory-based approach did not allow the investigators to obtain data on HIV status, clinical/radiological findings and TB treatment. A more comprehensive assessment of the success of diagnosing TB in children in Europe is needed. Ideally, new studies should be led by key organisations in the field of infectious diseases control and prevention, such as ECDC or WHO Regional Office for Europe, and result in effective recommendations.

As long as available evidence for paediatric TB diagnosis remains limited, however, this study is of public health importance as it reviewed routine laboratory work in a non-clinical trial context, allowing conclusions to be drawn based on real-life scenarios. Moreover, the study sites represent the largest laboratories with reference functions. This work has also demonstrated that data from the ERLN-TB can be used to conduct operational research.

Despite the relative success in diagnosing TB when using non-respiratory or GA samples, the level of laboratory confirmation in children remains low, resulting in treatment initiation based on a set of subjective parameters. TB treatment is long and antituberculosis drugs have potential toxicity; therefore while it is important not to risk failing to diagnose TB, overdiagnosis may result in unnecessary psychological or physical stress for children [26]. With this in mind, a more intensive approach to obtaining paediatric samples, including samples other than sputum (in particular GA and non-respiratory samples), is advisable in order to increase the number of laboratory-confirmed cases and give physicians a much greater degree of confidence when administering antituberculosis treatment. Additionally, a major effort is needed to optimise and evaluate molecular assays for analysis of GA or non-respiratory samples, thus making the diagnosis of paediatric TB more accurate.

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Conflict of interest

None declared.

Authors' contributions

Conceived and designed the study: AS, LF, FD, WH, CDM, YB. Collected data: AS, LF, WH, FD, ER, VKJ, EP, GS, CDM, ERLN-TB members, YB. Analysed the data: AS, LF, WH, FD, ER, VKJ, EP, GS, CDM, YB. Wrote the paper: AS, YB. All authors read and approved the final manuscript.

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