Original Article

Direct drug susceptibility testing of *Mycobacterium tuberculosis* against primary anti-TB drugs in northern India

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Abstract

Introduction: The present study aimed to evaluate a rapid and inexpensive colorimetric nitrate reductase assay (NRA) performed directly on sputum specimens for detection of drug resistance in *Mycobacterium tuberculosis* (MTB).

Methodology: A total of 55 sputum samples were decontaminated and processed by modified Petroff's method. A part of the resulting suspension was used to perform direct NRA (DNRA) and direct proportion method (DPM) analysis. Of the 55 samples, 45 could be used to compare the two methods. Indirect drug sensitivity testing (DST) was also done for 14 MTB strains.

Results: Excellent agreement was found between DNRA and DPM testing with κ values of 1, 0.91, 0.91, and 1 for RIF, INH, STR and EMB respectively. The sensitivities and specificities of DNRA compared to that of DPM were observed to be 100 and 100%, 100 and 93%, 95 and 96%, 100 and 100 % for RIF, INH, STR, and EMB respectively. Comparing the results of DNRA, DPM and indirect NRA with those of the gold standard indirect PM for 14 MTB strains showed that sensitivities, specificities and percent agreements were 100, 100 and 100% for all four tested drugs. Results for most of the specimens (55.6%) were available in 21 days with DNRA.

Conclusions: We have saved valuable time by omitting the pre-isolation step and conclude that DNRA is a rapid, accurate and inexpensive method for direct DST of MTB and may become an appropriate alternative method for the resource limited settings.

Introduction

Tuberculosis (TB) is contagious and spreads through air. People with active TB can infect on an average 10 to 15 people every year. One-third of the world's population (*i.e.*, about more than 2 billion people) is infected with tubercle bacilli [1], the bacteria that cause the deadly infectious TB disease. One in 10 people with latent TB infection will become sick with symptomatic TB infection in their lifetime. TB is a disease of poverty, and the majority of TB deaths occur in developing countries, affecting mostly young adults in their most productive years [2].

Multidrug-resistant TB (MDR-TB) is a form of TB that is difficult and expensive to treat because it fails to respond to two important first-line drugs, specifically, rifampicin (RIF) and isoniazid (INH). Data from more than 100 countries collected during the last decade show that 5% of all TB cases have MDR-TB. There were an estimated 500,000 new MDR-TB cases in 2007. Twenty-seven countries accounted for 85% of all MDR-TB cases. The top five countries with the largest number of MDR-TB cases are India, China, the Russian Federation, South Africa and Bangladesh, while extensively drug resistant TB (XDR-TB) has been found only in 58 countries to date [2,3].

The traditional method for detection of MDR TB with indirect susceptibility testing, involving isolation of the bacterium followed by drug susceptibility testing (DST), has a long turnaround time (TAT) of 10 to 12 weeks. Moreover, if the indirect DST is performed on solid medium, the TAT is longer. This long time required by the indirect methods may be a potential threat to patients, health workers, and the community [4]. While the use of liquid systems such as the radiometric BACTEC 460 TB system (Becton Dickinson, Sparks, MD, USA), the Mycobacteria Growth Indicator Tube (MGIT) 960 (Becton Dickinson, Cockeysville, Md.), BacT/ALERT 3D (bioMerieux, Durham, NC), or ESP Culture System II (Trek Diagnostics, Inc., Westlake, Ohio) has improved TAT to about 25–45 days [5-9], liquid culture systems require expensive substrates and equipment and are therefore not feasible in resource-poor settings [10].

Nitrate reductase assay (NRA) is described as a rapid, easily performed and inexpensive method for DST of *Mycobacterium tuberculosis* (MTB), which is based on the capability of MTB to reduce nitrate to nitrite [11]. However, since the test is performed indirectly (by using culture isolates of MTB), it takes three to four weeks more for the isolation of the same bacterium.

Considering the above problem, studies now focus upon direct drug susceptibility testing of MTB in which processed clinical specimens are directly inoculated in drug-free and drug-containing medium or amplified for detection of drug resistant TB. Some examples of the direct tests are nitrate reductase assay (NRA); microscopic observation drug susceptibility (MODS) assay; and molecular assays such as the Genotype MTBDR (Hain Life sciences, Nehren, Germany), and its newer version, the Genotype MTBDR*plus* [11-14]. Although a number of studies have been conducted to evaluate the performance of direct NRA [15-18], to the best of our knowledge only two are from India [19,20]. The present study was conducted to evaluate the performance of direct NRA in a region of northern India. Susceptibility tests were performed for all four first-line antitubercular drugs, *i.e.*, rifampicin (RIF), isoniazid (INH), streptomycin (STR) and ethambutol (EMB).

Methodology

Settings

This study was conducted in the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University. A total of 617 clinical sputum specimens of new and previously treated patients were collected from a tertiary referral hospital, Institute of Medical Sciences, Banaras Hindu University, as well as from two other TB centers of Varanasi for a period of seven months from July 2009 to January 2010.

Smear positive sputa/ Mycobacterial strains

Quality-assured bacteriological examination is an essential constituent for diagnosis and management of TB patients harbouring sensitive or resistant bacilli. Smear positive sputa were collected following World Health Organization (WHO) tuberculosis guidelines [21]. The smears were prepared and stained with the Ziehl-Neelsen technique [22,23]. A total of 55 smear-positive sputum samples with positive scores of 1+ (10-99/100 oil immersion fields) or more were evaluated in this study. Some scanty positive (1-9 bacilli/100 oil immersion fields) specimens were also included to check the sensitivity of the test. The sputum specimens were from cases reported for pulmonary TB. Indirect DST by NRA and proportion methods (PM) was performed for 14 strains; which had already been tested by direct DST methods.

Laboratory quality control

H37Rv (ATCC 27294) and *M. intracellulare* (ATCC 13950) strains served as nitrate positive and nitrate negative controls respectively. A known MDR strain was also used as a control.

Antitubercular drugs

INH, STR, RIF and EMB were obtained as powder from Sigma Aldrich (Bornem, Belgium). Each drug was prepared at a concentration of 10 mg/ml in sterile distilled water with the exception of RIF, which was dissolved in dimethylformamide (DMF). Stock solutions were filter (0.45 μm) sterilized and stored at −20°C for not more than one month.

Media

Conventional Lowenstein-Jensen (LJ) medium was prepared as described by Canetti *et al.* [24]. LJ medium for the NRA was prepared with a slight modification: 1mg/ml KNO_3 was added to LJ medium, with or without antibiotics, dissolved by stirring, and then aliquoted and inspissated once for 50 minutes at 80°C. After inspissation, the media were incubated for 48 hours at room temperature for sterility check before use. For NRA, the bottles containing LJ with antibiotics and KNO_3 were used in duplicate while LJ with KNO_3 were used as a control in triplicate for the reduction of the risk of contamination. One LJ bottle containing *p*-nitrobenzoic acid (PNB) was also used, along with a sensitivity test, as a part of routine biochemical testing for the identification of MTB.

Griess reagent

The following reagents were made in small volumes: 50% (vol/vol) concentrated hydrochloric acid (HCl), 0.2% (wt/vol) sulfanilamide, 0.1% (wt/vol) n-1-naphthylethylenediamine dihydrochloride. The reagents were mixed shortly

before use in the ratio: 1 part: 2 part: 2 part respectively.

Specimen digestion and decontamination

Modified Petroff's method was used to process sputum specimens [22,23] and thereafter concentrated by centrifugation at 3,200 × g for 20 minutes. The supernatant was discarded, and the sediment was re-suspended in 3 ml of sterile distilled water and then used to inoculate for culture as well as DST by direct NRA and direct PM. The isolated cultures from processed samples were then used for indirect DST by PM (the gold standard for DST of MTB) and NRA. They were also used for biochemical tests such as heat stable catalase, niacin accumulation, and susceptibility to PNB [25] for the characterization of MTB.

Direct DST by NRA

The direct NRA (DNRA) was performed according to Musa *et al.* [26] using Standard LJ medium with nitrate substrate, i.e., KNO_3 and with or without antibiotics. For LJ medium with antibiotics, the critical concentrations of 40 μg/ml for RIF, 0.2 μg/ml for INH, 2 μg/ml for EMB and 4 μg/ml for STR were used. Before NRA, part of the decontaminated suspension was diluted 1:10 in sterile distilled water. For each specimen, 0.2 ml of the undiluted suspension was inoculated into LJ medium containing nitrate substrate and antibiotics, and 0.2 ml of the 1:10 dilution was inoculated into three drug-free LJ medium tubes with KNO_3 incorporated. The latter tubes served as growth controls. The tubes were incubated at 37°C. After 10 days the Griess reaction was performed as described previously [11], for which 0.5 ml of freshly prepared Griess reagent was added to one drug-free tube. If any color appeared in the control tube, then reagent mixture were added to the tube with antibiotics. Otherwise, the other tubes were re-incubated, and the procedure was repeated on days 14, 18, and 28. Results were reported as positive if pink to violet color appeared in the medium. An isolate was considered resistant to a certain drug if there was a color change in the antibiotic tube in question greater than that in the 1:10 diluted growth control on the same day.

Direct DST by PM

The direct PM (DPM) on LJ medium without nitrate was performed according to Musa *et al.* [26] with the same recommended critical concentrations

of the four first-line antitubercular drugs used previously. For each strain, part of the suspension was diluted 1:100, and 200 μl of the dilution was inoculated on two tubes of LJ medium without antibiotics and 200 μl of the undiluted suspension was inoculated into two LJ medium tubes with antibiotics incorporated. All tubes were incubated at 37°C. Preliminary results could be reported earlier for resistant strains, sometimes as early as after 20 days. Final susceptibility results were reported only after 40 days following the standard procedure [22,23]. An isolate was reported as resistant if the number of colonies growing on the antibiotic containing medium was 1% or more of the number of colonies developing on the drug-free control. The results obtained by the proportion method were used as the gold standard to compare to the results of NRA for susceptibility testing.

Indirect DST by PM

The Indirect PM (IPM) was performed on LJ medium with the same recommended critical concentrations of antibiotics as mentioned previously. Briefly, bacterial suspensions for DST were prepared by adding approximately 4 mg (2/3 loopful 3 mm internal diameter 24 SWG wire loop) of moist weight of a representative sample of bacterial mass in 200 μl of sterile distilled water in a bijou bottle with 4 to 5 glass beads, then vortexed for about 30 seconds to obtain a uniform solution. In order to obtain 1 mg/ml suspension, 3.8 ml sterile distilled water was then added to the bottle and allowed to settle for about 30 minutes before gently aspirating the upper portion into a fresh bijou bottle (S1 suspension). S1 was further diluted 10-fold to obtain S2-S4. S1-S4 bacterial concentrations were respectively inoculated into drug-free and drug-containing LJ slopes using a 3 mm internal diameter wire loop and incubated at 37°C. Growth was recorded at 28 days and at 42 days as follows: +++ for confluent growth, ++ for more than 100 colonies, and 1-100 actual number of colonies. Susceptibility or resistance was recorded when the proportion of bacteria in drug-containing medium to that of drug free medium was < 1 or ≥ 1 respectively.

Indirect DST by NRA

Indirect NRA (INRA), also called the Griess method, was performed according to the method described by Angeby *et al.* [11] with the same

recommended critical concentration of the four first-line antitubercular drugs [22].

Analysis of data

MedCalc Software (MedCalc, Mariakerke, Belgium) was used to calculate the statistical parameters, sensitivity and specificity. Predictive values were calculated by using the prevalence of RIF, INH, STR, and EMB resistance in all TB cases in Varanasi, India. The agreement between NRA with the standard PM was determined by the κ statistic. The κ value, a measure of test reliability, was interpreted as follows: < 0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥ 0.81, excellent [27]. The standard error was also estimated to check the reproducibility of the test.

Results

Direct drug sensitivity testing for the first-line antitubercular drugs RIF, INH, STR and EMB was performed on 55 clinical sputum specimens using direct NRA and PM testing. Of these 55 specimens, only 45 could be used for the DNRA and DPM comparison (11 specimens had 1+ acid-fast bacilli positivity; 11 had 2+ positivity; 21 had 3+ positivity; and 2 had scanty positivity with 8 and 3 bacilli load/100 oil immersion field). The remaining samples were culture negative (5), identified as non-tubercle mycobacteria (3) or were contaminated (2). (See Table 1 for details). The results of 25 (55.6%), 11 (24.4%) and 9 (20%) isolates were obtained after 21, 25 and 28 days respectively with DNRA and were compared with the results produced using DPM that were obtained after 28 days for 4 (8.88%) isolates and 40 days for 41 (91.11%) isolates. The results are given in Table 2.

For RIF, 20 and 25 strains were detected as resistant and susceptible respectively by means of both methods. For INH, both methods detected 17 resistant strains and 26 susceptible strains, while two false positive results were obtained with the NRA. For EMB, 16 strains and 29 strains were identified

true positive and true negative respectively by both the methods used. For STR, 19 isolates were correctly detected as resistant and 24 as sensitive by both methods; two isolates gave discrepant results; one was false negative, resistant by PM while being susceptible by NRA; and one isolate was false positive, susceptible by PM but resistant by NRA. Excellent agreement was found between two tests with κ values of 1, 0.91, 0.91, and 1 for RIF, INH, STR, and EMB respectively. The sensitivities and specificities of DNRA compared to those of DPM were observed to be 100 and 100, 100 and 93, 95 and 96, 100 and 100% for RIF, INH, STR, and EMB respectively. Positive predictive values were 100, 89, 95 and 100% for RIF, INH, STR and EMB respectively. Negative values were 100, 100, 96 and 100% for RIF, INH, STR and EMB respectively. Standard error was very small in the results for RIF, INH, STR and EMB, *i.e.*, ± 0.1491, ± 0.1484, ± 0.1491 and ± 0.1491 respectively, indicating very little chance of disagreement between the results of DNRA and DPM, and the reproducibility of the assay (Table 2).

MTB growths from plain drug-free LJ slopes, from the cases of direct DST by PM, were used for indirect DST. Indirect DST by NRA and PM methods was performed for 14 strains, of which 4, 3 and 6 sputum specimens had 1+, 2+ and 3+ microscopy positivity respectively. One was a scanty positive sputum sample with 8 AFB load/100 oil immersion field. By comparing the results of DNRA, DPM, and INRA with those of the gold standard IPM for 14 MTB strains, the sensitivities, specificities, positive predictive values, negative predictive values, and percent agreement were 100, 100, 100, 100 and 100% against all four tested drugs. The results of five (35.71%) and nine (64.28%) isolates were obtained after 10 and 14 days respectively. Results with INRA and with standard IPM were obtained after 28 days with three (21.42%) isolates and 42 days with 11 (78.57%) isolates.

Table 1. Comparison of direct and the indirect susceptibility methods on the basis of bacillary counts

Bacillary count*	Nitrate reductase assay (NRA)				Proportion Method (PM)		
	No. Tested	NTM	Direct NRA available (n = 45)	Performed Indirect NRA (n = 14)	No. Tested	Direct PM available (n = 45)	Performed Indirect PM (n = 14)
1+	14	1	11	4	14	11	4
2+	12	1	11	3	12	11	3
3+	26	1	21	6	26	21	6
Scanty+	3	0	2	1	3	2	1
Total	55	3	45	14	55	45	14

* 1+ (10–99 bacilli per 100 fields examined), 2+ (an average of 1–10 bacilli per field in 50 examined fields), 3+ (an average of >10 bacilli per field in 20 examined fields), Scanty+= (1-9 bacilli per 100 oil immersion field), TAT = Turnaround time; NTM = Non-tubercle mycobacteria

Table 2. Comparison of the results of direct drug susceptibility testing of 45 clinical sputum specimens by NRA and PM

Antitubercular Drugs	Direct proportion Method	Direct NRA							
		No.		%				Agreement (kappa value)	SE
		Resistance	Susceptible	Sensitivity	Specificity	Negative predictive value	Positive predictive value		
RIFAMPICIN	Resistance Susceptible	20 0	0 25	100	100	100	100	1	± 0.1491
ISONIAZID	Resistance Susceptible	17 2	0 26	100	93	100	89	0.91	± 0.1484
STREPTOMYCIN	Resistance Susceptible	19 1	1 24	95	96	96	95	0.91	± 0.1491
ETHAMBUTOL	Resistance Susceptible	16 0	0 29	100	100	100	100	1	± 0.1491

SE = Standard error

Table 3. Published studies using direct nitrate reductase assay for drug susceptibility testing of *M. tuberculosis*

Study	Setting	No. of samples compared/ Media	Antibiotics	Sensitivity (%)	Specificity (%)	Agreement (%)	TAT
Musa *et al.* (2005)	Mycobacteriology referral center, Buenos Aires, Argentina	121/Solid	RIF, INH, STR and EMB	100, 93, 76 and 55 respectively	100%, 100%, 100%, 99% respectively.	An overall agreement of 98%	14 days
Solis *et al.* (2005)	The National Institute of Health, Peru	192/Solid	INH, RIF	99.1 and 93.5% respectively	100 and 100% respectively	99.5 and 96.4% for INH and RIF respectively	28 days
Affolabi *et al.* (2007)	Mycobacteriology reference laboratory, Benin	213/Solid	RIF	87.5	100	93%	18 days
Affolabi *et al.* (2008)	Mycobacteriology reference laboratory, Benin	144/Liquid	INH, RIF	100 & 100 respectively	99.2 & 99.3% respectively	91 and 96% respectively	10 days
Mishra et al (2009)	Primary care center, India	32/Solid	INH, RIF	100 & 100 respectively	100 & 100% respectively	87.5% for INH and 97% for RIF	21
Visalakshi *et al.* (2009)	Tertiary care centre, India	108/Solid	INH, RIF	100 and 97.91% respectively	93.75 and 100% respectively	97.22% for INH and 99.07% for RIF	21
Shikama *et al.* (2009)	Mycobacteria Reference Laboratory, Brazil.	210/Solid	RIF	100	100	100	15
Agatha *et al.* (2009)	TB centres, Jos, Nigeria,	10/Solid	RIF, INH, STR and EMB	--	--	90%	10-14
Present Study (2010)	Tertiary care centre, India	45/Solid	RIF, INH, STR and EMB	100, 100, 95 and 100 respectively	100, 93, 96 and 100 respectively	100, 91, 91 and 100 respectively	21-28 days

TAT: Turnaround time

Discussion

Complete agreement for the results of the DNRA and DPM was seen against RIF and EMB and an excellent agreement was also found in the results among the same for STR and INH. In the cases of INRA and IPM, full agreement was found against all four tested drugs. Instead of focusing only on RIF and INH, the present study focused upon all four first-line anti-TB drugs, *i.e.* RIF, INH, STR and EMB. Current methods for DST of MTB are either expensive or have a long TAT; therefore, a cost-effective and rapid drug susceptibility method is required to guide tuberculosis treatment.

In our study direct DST was performed for 55 clinical sputum specimens with a positivity score of 1+ or more. In addition, four scanty positive specimens were also used to check the sensitivity of the test and we achieved good results. Out of four scanty positive specimens, two were available for the tests while two were culture negative. An interesting finding about these two specimens was that one had only a 3 AFB load/100 oil immersion field while the other had an 8 AFB load. So we could assume that if the specimens were carefully processed, then we were capable of achieving good results even with a lesser number of bacterial loads. DNRA, DPM, INRA and IPM were performed for the same specimens and we found full agreement. Of 55 samples, only 45 specimens could be used for the comparison between DNRA and DPM, while five,

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three, and two were identified as culture negative, non-tubercle mycobacteria (NTM), and contaminated respectively. Most of the culture negative specimens had a 3+ positivity score, which was probably due to excessive treatment of NaOH during the sample processing. NTM were identified initially when readings of DNRA (absence of color) and DPM (growth on PNB containing LJ-slopes) were taken and further confirmed by growth characteristics, pigmentations, and certain biochemical tests. Our results for all four used drugs were also similar to those obtained by other authors with the indirect NRA [28,29] and also comparable with direct NRA [18,20]. We have compared the DNRA assay with those of DPM as Musa *et al.* [26] and INRA with the internationally accepted gold standard IPM.

The NRA method utilized nitrite as the indicator of growth and results were observed much earlier than using visible growth as an indicator. Since the assay used clinical specimens, we can decrease the six to eight weeks normally required for the isolation of the bacilli. Affolabi *et al.* [16] found that a liquid medium-based NRA further reduced the TAT with clinical sputum specimens as 56% of their results were obtained in 10 days.

In a meta-analysis of direct susceptibility testing for MDR-TB by Bwanga *et al.* [14], the sensitivity, specificity, and time to results of four direct DST tests were compared with the conventional indirect testing for detection of resistance to RIF and INH in MTB. NRA was one of the four direct tests used and showed sensitivity and specificity to RIF of 99% and 100% and to INH of 94% and 100%.

Syre *et al.* [30] used the colorimetric nitrate reductase-based antibiotic susceptibility (CONRAS) test for DST of MTB against INH and RIF in liquid cultures. The results were produced within five days, indicating that the CONRAS test was an alternative in all settings, particularly for resource-poor countries.

Ani *et al.* [17] used both direct and indirect methods in his study. The indirect NRA showed sensitivity and specificity for INH: 100% and 100%, EMB: 75% and 100%, RIF: 90% and 96.6%, STR: 66.6% and 91.8%. The results of DNRA and PM for INH, EMB, RIF and STR agreed 10/10 (100%) for AFB negative specimens and 9/10 (90%) with AFB positive specimens.

In a multicentric study [31], the performance of INRA was evaluated in different settings to ascertain the susceptibility of MTB to first-line antitubercular drugs. The accuracy was greater than 97% for INH, EMB and RIF while that for STR was inferior (85.3%). Furthermore, Martin *et al.* [32] reported the evaluation of NRA for ofloxacin, a second-line drug, and found complete agreement with the agar PM. Therefore, NRA also has the capability to be used for the evaluation of second-line drugs. In addition, Lemus *et al.* [33] evaluated indirect NRA with 320 strains of MTB and found an overall 98.8 % agreement between the INRA and IPM.

Khan and Sarkar aimed to develop a whole cell based, high-throughput screening protocol to identify inhibitors against both active and dormant TB bacilli, based on the principle of the induction of a respiratory type of nitrate reductase (NarGHJI) during dormancy that can reflect the viability of dormant bacilli of *Mycobacterium bovis* BCG in a microplate adopted model of *in vitro* dormancy. They found a good agreement between NRA and BACTEC and bioluminescence screening *In Vitro* (Bio-Siv) assays. With S/N ratio and Z' factor of 8.5 and 0.81 respectively for the assay, they concluded that it provides an inexpensive, robust and high-content screening tool to search novel antitubercular molecules against both active and dormant bacilli [34].

A full agreement was observed for RIF resistance detection while some discordant results were obtained for other drugs in the study of Musa *et al.* [26]. Visalakshi *et al.* [20] observed sensitivity and specificity of the DNRA and IPM to be 94% and 98%, and 100% and 98% for RIF and INH respectively. Additionally, Shikama *et al.* [35] stated 100% sensitivity and specificity of NRA for RIF with a TAT of 15 days. In another study, Shikama *et al.* [18] found the reproducibility of NRA was 100% for INH and EMB and 97% for STR and RIF. Percent agreement between the results of NRA and PM was superb for INH and RIF, *i.e.*, 98.3%. The meta-analysis by Martin *et al.* [36] of NRA suggests that the NRA is highly sensitive and specific for determining RIF- and INH-resistant TB in both culture isolates and directly on clinical sputum specimens. Most of the studies had a sensitivity of 95% or greater, and nearly all were 100% specific with high degree of accuracy. The average TAT was between 5 and 12 days with indirect NRA, and 14 and 21 days with direct NRA. The biggest asset of NRA is that there is no need to change laboratory infrastructure as it is performed in classical LJ medium, routinely used in TB laboratories, with the

addition of KNO_3. There is no need for any sophisticated equipment or expensive reagents, making it widely accessible method. Results are easy to observe by a color change of the medium.

The basis of the NRA test is the reduction of measurable nitrate by metabolic activity of MTB cells. The isolates, resistant to certain anti-TB drugs, may have lower metabolic activity [11] (for example, *rpo*B and *kat*G mutations), leading to RIF and INH resistance, which might affect the expression levels of nitro reductase enzymes. These strains could have a low level of resistance that could not be detected by the NRA. By keeping in mind the results of various studies (as shown in Table 3), we have concluded that the direct NRA has the potential to be an inexpensive alternative method for DST of MTB. As this assay is applied directly, it may also be useful to reduce the burden of the laboratories. However, further studies are necessary with a larger number of specimens.

Acknowledgements

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