Interferon Gamma Release Assay, A Powerful Tool for the Detection of Human and Bovine Tuberculosis in the Greater Cairo Area Compared to Other Diagnostic Tools
(Asai Pelepasan Gama Interferon, Suatu Alat Penting untuk Pengesanan Tuberkulosis Manusia dan Bovin di Kawasan Kaherah Besar Berbanding dengan Alat Diagnostik Lain)

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ABSTRACT

Rapid detection is essential for the elimination and control of tuberculosis (TB) worldwide. Our study aimed to show the current and actual patterns of human and bovine TB distribution in the Greater Cairo Area community by the application of different TB diagnostic tools to individuals and farm animals with suspected TB. Both sputum and blood specimens were collected from 150 suspected human cases in the community. Sputum samples were examined using direct microscopy (Ziehl-Neelsen stain), culture on Lowenstein-Jensen medium, and real-time PCR. Blood samples were used for interferon gamma release assay (IGRA). In addition, lymph nodes and blood samples were collected from 57 tuberculin-positive animals. Lymph nodes were examined using direct microscopy (Ziehl-Neelsen stain), culture on Lowenstein-Jensen medium, and real-time PCR. Animal blood samples were also tested with IGRA. Sensitivity and specificity as well as positive and negative predictive values were calculated for all tests. The results showed that for both human and animal samples, IGRA provided the most accurate estimates of current TB infection compared to other tests. Furthermore, IGRA had the highest sensitivity and was the most convenient, proving its superiority compared to traditional methods in showing true levels of TB dissemination. This work shows that IGRA is a powerful tool for detection of TB in suspected humans and farm animals and should be incorporated into routine TB screening programs, which require more than one test.

Keywords: Egypt; interferon gamma release assay; Mycobacterium species; TB Real-time PCR

ABSTRAK

Pengesanan pantas adalah penting untuk penghapusan dan pengawalan tuberkulosis (TB) di seluruh dunia. Kajian kami bertujuan untuk menunjukkan corak terkini dan sebenar taburan TB manusia dan bovin di komuniti kawasan Kaherah Besar dengan penerapan alat diagnostik TB yang berbeza kepada individu dan haiwan ternakan yang disyaki TB. Kedua-dua spesimen kahak dan darah dikumpulkan daripada 150 kes manusia yang disyaki dalam kalangan masyarakat. Sampel kahak diperiksa menggunakan mikroskopi langsung (stain Ziehl-Neelsen), kultur pada medium Lowenstein-Jensen dan PCR masa nyata. Sampel darah digunakan untuk asai pelepasan gama interferon (IGRA). Sebagai tambahan, nodus limfa dan sampel darah dikumpulkan daripada 57 haiwan positif tuberkulin. Nodus limfa diperiksa menggunakan mikroskopi langsung (stain Ziehl-Neelsen), dikultur pada medium Lowenstein-Jensen dan PCR masa nyata. Sampel darah haiwan juga diuji dengan IGRA. Kesensitifan dan kekhususan serta nilai ramalan positif dan negatif dihitung untuk semua ujian. Hasil kajian menunjukkan bahawa untuk sampel manusia dan haiwan, IGRA memberikan anggaran yang paling tepat mengenai jangkitan TB semasa berbanding dengan ujian lain. Selanjutnya, IGRA mempunyai kesensitifan tertinggi dan paling mudah, membuktikan keunggulannya dibandingkan dengan kaedah tradisi dalam menunjukkan tahap penyebaran TB yang sebenarnya. Kertas ini menunjukkan bahawa IGRA adalah alat penting untuk mengesan TB pada manusia dan haiwan ternakan yang disyaki dan harus dimasukkan ke dalam program pemeriksaan rutin TB, yang memerlukan lebih daripada satu ujian.

Kata kunci: Asai pelepasan gama interferon; Mesir; Mycobacterium species; PCR masa-nyata TB

INTRODUCTION

Tuberculosis (TB) is one of the most resilient infectious diseases globally, despite the existence of a live attenuated vaccine (BCG) as well as several potent antimicrobials (Issar 2003). Detection of *Mycobacterium tuberculosis* infection is of great importance for the elimination and control of TB worldwide; however, the existing screening methods are still limited (Ai et al. 2016). The World Health Organization (WHO) reports that approximately 2-3 billion people in the world are latently infected with *M. tuberculosis*, and 5%-15% of these people will develop active disease during their lifetime. In Egypt, the incidence of TB is estimated at 15 per 100,000 population per year. Accordingly, TB is considered to be the third most

important communicable disease problem in Egypt, after schistosomiasis and hepatitis C, despite the low levels of HIV infection (WHO 2015).

Mycobacterium tuberculosis complex (MTBC) is a group of genetically related pathogens that cause TB in mammals. *M. tuberculosis* and *M. bovis* are the most common human- and animal-adapted strains, respectively (Gordon & Marcel 2015). *M. tuberculosis* may cause active or latent TB infection. The latter is a reservoir for future epidemics, as many risk factors may lead to the development of active disease including malnutrition, acute infections, or decreased immunity. *M. bovis* is a pathogen of significant importance in livestock and farm animals. It is also known to cause zoonotic TB in humans, raising concerns regarding the zoonotic risk for those living at the animal-human interface (Müller 2009). Zoonotic transmission of these pathogens occurs through close contact with infected cattle or consumption of contaminated animal products such as unpasteurized milk (Ayele et al. 2004; Cosivi et al. 1998; Müller et al. 2013). In Africa, although bovine TB is prevalent, effective disease control-including regular milk pasteurization and slaughterhouse meat inspection-is largely absent (Cosivi et al. 1998).

The identification of people with latent TB infection (LTBI) is one of the first priorities of TB control programs (Broekmans et al. 2002) to prevent development of active disease (Anderson et al. 2000). There is no gold standard for the detection of LTBI, although the tuberculin skin test (TST), which uses purified-protein derivative (PPD), is available (Lalvani 2007). The TST has many limitations, as the PPD antigens used for the TST are also present in the BCG vaccine strain, and false positive tests are thus common. Additionally, immunocompromised patients give false negative results and repeated visits are needed, rendering it inconvenient for screening large number of patients or screening farm animals (American Thoracic Society 2000).

The conventional sputum smear microscopy method of detection remains the only diagnostic tool available in some high-burden countries, and although it can detect 60%-80% of culture-positive TB cases under optimal conditions, the sensitivity is as low as 10%-30% in HIV-positive populations. In addition, it cannot be used to distinguish between various members of the family Mycobacteriaceae (Patama et al. 2009). Detection of *Mycobacteria* spp. using the conventional culture method is tedious and time consuming, and the decontamination process presents additional obstacles.

Novel methods for the detection of mycobacteria that can provide results within 1-2 days and be performed directly on sputum and other clinical samples are continuously being developed (Hassan et al. 2014). Interferon gamma (IFN-γ) release assay (IGRA) is an in vitro measurement of the cell-mediated immune response to *M. tuberculosis* infection. If re-exposed, lymphocytes previously exposed to *M. tuberculosis*-specific antigens release IFN-γ, which can be measured as a method of detecting TB infection (past or current) (Mazurek et al. 2010). IGRAs overcome many drawbacks of the TST, including enhanced specificity and ease of application (Gormley et al. 2004).

Our study compares different diagnostic tools to reveal the most reliable method for detection of the current distribution rates of TB in the Egyptian community. Application of a reliable and powerful diagnostic tool will lead to the prompt choice and initiation of the most appropriate antimicrobial therapy.

MATERIALS AND METHODS

STATEMENT OF ETHICAL APPROVAL

Sample collection and all experiments were conducted in accordance and approval of the Safety and Occupational Health Committee of the Faculty of Pharmacy at Cairo University, Cairo, Egypt, which approved the protocol as a whole and the sample collection schemes (Approval # MI 1370, 27th May 2015). All specimens had previously been collected by routine monitoring laboratories, and no direct contact was made between any of the authors and any human or animal subjects.

A total of 150 sputum (three consecutive samples taken in the morning then pooled) and blood samples were collected from 150 suspected human cases as follows: 80 community patients (HIV and cancer patients and samples from hospital laboratories), 50 casual contacts (health care workers in clinics and laboratories) and 20 people who had contact with farm animals (farm workers, veterinarians, slaughter house workers). In addition, 57 lymph node (LN) samples and 57 blood samples were collected from tuberculin-positive animals just before slaughter.

All samples were collected during the period from March 2016 to March 2017 in locations distributed within the Greater Cairo Area. Great Cairo is a large urban area that comprises Cairo-the capital of Egypt-Giza, Helwan, Shoubra El Kheima and all of their surrounding suburbs. The population of the Greater Cairo Area exceeds 20 million. Human sputum samples were prepared and examined using direct microscopy after Ziehl-Neelsen staining (ZN smear), culture on Lowenstein-Jensen (LJ) media, and real-time PCR. Human blood samples were used for detection of interferon gamma release. For the animal subjects, both lymph nodes and blood samples were collected from 57 tuberculin-positive animals. Lymph nodes were examined using direct microscopy, culture and real-time PCR, and blood samples were tested using IGRA.

DIRECT MICROSCOPY AND CULTURE TECHNIQUE

A direct smear was done after staining with Ziehl-Neelsen stain for the 150 sputum samples from persons with suspected TB, and a digestion-decontamination

procedure was then performed on every sputum specimen. LJ slants were inoculated with 3 drops of neutralized sediment. The cultured tubes were examined daily for a week then once per week for 6-8 weeks (Petroff 1915). Regarding the animal samples, the infected lymph nodes of 57 tuberculin-positive cows were prepared for direct microscopy and culture on LJ media as previously described (Marks 1972; Ratledge & Stanford 1982).

MOLECULAR DETECTION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX USING REAL-TIME PCR

A The total collection of 150 sputum samples from persons with suspected TB, in addition to the infected LN samples of 57 tuberculin-positive cows, were examined using real-time PCR. Preparation of animal LN samples was done as previously described (Ward et al. 1995), and then DNA extraction was done following the instructions of the extraction kit (Sigma Aldrich, USA). Real-time PCR was performed to detect the MBTC as previously described using the MTplex dtec-RT-qPCR test kit (Edifici-Quórum3, Spain) (Ben Kahla et al. 2011). The kit comprises a series of species-specific targeted reagents designed for detection of all species contained in the MBTC. The genomic marker was the repetitive element IS6110, the PCR product size was 254 bp, and the forward primer 5′-CGTGAGGGCATCGAGGTGGC-3′ and reverse primer 5'- GCGTAGGCGTCGGTGACAAA-3' were used as previously described (Van et al. 1991). The specific primers used to detect *M. bovis* were the forward primer 5'-CAGGGATCCACCATGTTCTTAGCGGGTTG-3' and reverse primer 5'-TGGCGAATTCTTACTGTGCCGGGGG-3' as previously described (Jiang et al. 2006), while the specific primers used to detect *M. tuberculosis* (human type) were the forward primer 5'-GCCTCGAACAATCCGGEGTTGACAA'3 and reverse primer 5'-GAGGCATTAGCACGCTGTCAATC-3'.

Extracted DNA from the suspected samples was subjected to real-time PCR. The reaction volume consisted of 10 µl Hot Start-Mix qPCR 2×, 1 µl MTplex dtec-qPCR-mix, 5 µl of DNA sample, and DNase/RNase-free water to 20 µl. The reaction conditions consisted of one cycle of 95 ºC for 5 min followed by 45 cycles of 95 ºC for 30 s and 60 ºC for 60 s for hybridization, extension, and data collection. The reaction was run in an Applied Biosystem StepOne Real-time PCR System (Applied Biosystem, USA), and FAM fluorogenic signal was collected and the cycle threshold (CT) of the reactions was detected by StepOne™ software version 2.2.2 (Life Technology, USA). The CT was defined as 10 times the standard deviation of the mean baseline fluorescence emission calculated for PCR cycles 3-15. For a sample to be considered positive, the corresponding amplification curve had to exhibit three distinct phases (geometric, linear, and plateau) that characterize the progression of the PCR reaction.

IGRA

Sample The 150 whole blood samples from suspected human cases were subjected to the QuantiFERON-TB-Gold In-Tube assay (QFT-GIT) (Cellestis Ltd, Victoria, Australia) as follows. A blood sample was collected into 3 tubes; the first tube was coated with TB-specific antigens, the second with heparin-negative control, and the third with mitogen (phytohaemagglutinin) as positive control. Blood was incubated overnight at 37 °C. Plasma was then separated to measure the concentration of IFN-γ by enzyme-linked immunosorbent assay using the QFT-GIT assay (Cellestis Ltd, Victoria, Australia) according to the manufacturer's instructions (Abu-Taleb et al. 2011).

Animal blood samples were subjected to the *M. bovis* gamma interferon test kit for cattle, Bovigam (Prionics, Germany), as follows. For each sample, 5 mL of blood was collected into heparinized blood-collecting tubes. Samples were transported to the laboratory within 12 h. Aliquots of 1.5 mL of heparinized blood from each sample were dispensed into 24-well tissue culture trays. An aliquot of 100 µl of PBS (nil antigen control), avian PPD (purified protein derivative) or bovine PPD were added to the appropriate 3 wells containing the tested blood. The tissue culture trays containing blood and antigens were incubated for 16-24 h at 37 °C. Plasma was then separated to measure the concentration of IFN-γ by enzyme-linked immunosorbent assay using the Bovigam kit (Prionics, Germany) following the manufacturer's instructions.

FINAL DIAGNOSIS

The final diagnosis was obtained from the patient´s medical record. It was made on the basis of all clinical, radiological, microbiological, and pathological information gathered.

STATISTICAL ANALYSIS

Sensitivity, specificity, positive predicted value (PPV), and negative predicted value (NPV) were calculated for each diagnostic test, compared to the final diagnosis of TB. Statistical calculations were performed to compare the sensitivity and specificity of the TB detection methods.

RESULTS

MICROSCOPIC EXAMINATION, CULTURE, REAL-TIME PCR AND IGRA OF HUMAN AND ANIMAL SAMPLES

As shown in Table 1, the microscopic examination of 150 human sputum samples collected from persons with suspected TB showed that 37 samples (25%) harbored the acid-fast bacilli. The culture method yielded 43 isolates of *Mycobacterium* spp. (29%), while real-time PCR detected 49 positive cases (33%), which were identified as MBTC. Results of IGRA showed that 59 blood samples (39%) were positive for TB.

On the other hand, microscopic examination of lymph node of infected animals showed that 31 samples (54%) harbored the acid-fast bacilli, while the culture method yielded 37 isolates of *Mycobacterium* spp. (65%) after 6-8 weeks of incubation, and real-time PCR and IGRA detected 53 samples (93%) that were positive for *M. bovis* (Table 1).

In both human and animal subjects, all positive TB samples detected by the conventional methods (microscopy and culture) were also detected as positive using real-time PCR and IGRA.

SAMPLE DIVERSITY

As for the diversity of the 49 real-time PCR-positive human isolates (+ve MBTC), the results of species-specific PCR showed that 46 isolates belonged to the human type (*M. tuberculosis*), while 3 isolates were identified as *M. bovis* (Table 2). Only *Mycobacetrium tuberculosis* isolates were detected in community patients (31/80; 39%) and casual contacts (14/50; 28%). Both *M. tuberculosis* and *M. bovis* were isolated from farm animal workers (4/20; 20%), where *M. tuberculosis* represented 1/20 (5%) and *M. bovis* represented 3/20 (15%) of the isolates.

For animal samples, all of the 53 real-time PCR-positive isolates (+ve MTBC) were identified as *M. bovis*, as shown in Table 2.

STATISTICAL ANALYSIS

When diagnostic tests were applied to the samples of the study group, the results were distributed as shown in Table 3. The sensitivity and specificity of the ZN smears were 74% and 100%, respectively. In addition, the ZN smear had a PPV of 100% and an NPV of 88.5%. Culture on LJ medium had sensitivity and specificity of 84% and 99%, respectively, and PPV and NPV of 97.67% and 92.52%, respectively. Real-time PCR had a sensitivity of 92% and specificity of 97%, a PPV of 93.88%, and an NPV of 96.04%. IGRA had a sensitivity of 96%, a specificity of 89%, PPV of 81.36% and NPV of 97.8%.

Fifty cases were diagnosed as TB patients according to patients' medical records, of which 48 tested positive and 2 negative by IGRA, 37 positive and 13 negative by ZN smear, 42 positive and 8 negative by LJ culture, and 46 positive and 4 negative by real-time PCR. Meanwhile, 11 out of 100 participants diagnosed as non-TB patients were IGRA positive and 3 were real-time PCR-positive. Regarding animal samples, the results were distributed as shown in Table 4. The sensitivity of the ZN smears was 54%, culture sensitivity was 65%, and real-time PCR and IGRA both had a sensitivity of 93%.

TABLE 1. Detection of *Mycobacterium* species using different diagnostic tools

	Source	No. of samples	Bacteriological findings		Molecular findings	Blood based Assay findings
			Microscopy	Culture	Real-time PCR	IGRA
			No. of isolates (%)	No. of isolates (%)	No. of isolates (%)	No. of isolates (%)
Human	Sputum	150	37(24.67)	43 (28.67)	49 (32.67)	-
Human	Blood	150	-	-	-	59 (39.33)
Animal samples	Lymph nodes	57 tuberculin+	31 (54.39)	37 (64.91)	53 (92.98)	-
Animal samples	Blood	57 tuberculin +	-	-	-	53 (92.98)

TABLE 2. Diversity of human and animal samples

Source	Human samples			
	Total no. of samples	No. of +ve LTB cases using real-time PCR (%)	No. of *M. tuberculosis* (%)	No. of *M. bovis* (%)
Community patients	80	31(39)	31 (100)	0
Casual contacts	50	14(28)	14 (100)	0
Farm animals contacts	20	4 (20)	1(25)	3(75)
Total	150	49(32.67)		3
Animal samples				
Total	57	53(93)	0	53(100)

TABLE 3. Sensitivities, specificities, PPVs and NPVs of ZN smear, culture, real-time PCR and IGRA tests in TB suspected human patients recruited in this study, n = 150

Method of diagnosis	Negative (%)	Positive (%)	Sensitivity %	Specificity %	PPVa, %	NPVa, %
ZN Smear [b]	113 (75.33)	37 (24.67)	74	100	100	88.5
Culture [c]	107 (71.33)	43 (28.67)	84	99	97.67	92.52
Real-time PCR [d]	101 (67.33)	49 (32.67)	92	97	93.88	96.04
IGRA [e]	91 (60.67)	59 (39.33)	96	89	81.36	97.8
Diagnosis [f]	100 (66.67)	50 (33.33)				

a PPV, Positive predictive value; NPV, Negative predictive value. b Smears of respiratory samples were stained by Ziehl neelsen method for the detection of AFB (mycobacteria species). c Lowenstein Jensen's media was used for mycobacteria culture of respiratory samples. d Real-time PCR = Real-time polymerase chain reaction targeting both the multicopy target IS6110 insertion element and a common genomic *subsequence* on respiratory samples. e IGRA = Interferon gamma releasing assay in whole blood performed in suspected active TB patients to detect the exposure to MTB (results above the cutoff 0.35 IU/ml were considered as positive). f Final diagnosis taken from patient´s medical record.

TABLE 4. Sensitivity of ZN smear, culture, real-time PCR and IGRA tests in tuberculin positive animals' samples n=57

Method of diagnosis	Negative	Positive	Sensitivity
ZN smear	26	31	54.39 %
Culture	20	37	64.91 %
Real-time PCR	4	53	92.98 %
IGRA	4	53	92.98%

DISCUSSION

The objective of the present study was to estimate the expediency of an IFN-γ assay for the rapid detection of mycobacterial TB infection in suspected individuals and farm animals and to conduct a comparative study between conventional, molecular methods and IGRA to identify the most appropriate method for detecting TB distribution in the Greater Cairo Community. Sputum and blood samples were collected from a total of 150 suspected individuals. Sputum samples were examined using traditional methods. Blood samples were used for detection of gamma interferon release using the QuantiFERON-TB-Gold In-Tube assay (QFT-GIT). In addition to the human samples, lymph nodes and blood samples were collected from 57 tuberculin-positive animals. Lymph nodes were examined using microscopy and culture and the results confirmed by real-time PCR, while blood samples were tested for IGRA using the Bovigam assay.

In both human and bovine subjects, IGRA was able to detect higher percentages of TB infection cases than conventional methods or real-time PCR. All cases of TB detected by the conventional methods (microscopy with ZN stain and culture) were positive using real-time PCR and IGRA.

The prevalence of LTBI caused by *M. tuberculosis* among the high-risk community patients as well as health care workers (Table 2) was similar to that found in previous studies carried out in Egypt and the Middle East (El-Sokkary et al. 2015; Hiban & Hasan 2015). Such levels of infection prevalence are relatively high despite the relatively low rates of HIV infection and show that a standard program for detection and treatment of LTBI should be encouraged in the Middle East.

M. bovis (bovine TB) infections were detected in 15% (3/20) of subject with farm animals contact (Table 3). This is in agreement with previous studies and confirms the need for control programs to prevent and screen human infections resulting from animal

encounters (Cosivi et al. 1998; Hiban & Hasan 2015; Taylor et al. 2007). The viability of *M. bovis* in animal organs is essential for the isolation of mycobacteria by culture-based methods. The viability may be reduced by either freezing/unfreezing practices or by treatment with the selected decontaminant reagent, which is not completely innocuous for *M. bovis*. In addition, many factors influence the success of primary isolation of *M. bovis* from clinical specimens, including the culture media used, the decontamination procedures, and how samples are obtained and handled.

Although the isolation of mycobacteria and their identification based on phenotypical characters, particularly culture, has been used as a gold standard, these traditional methods are time consuming; consequently, their use is on the decline and they are being replaced by other diagnostic tools. In our study, the results obtained from real-time PCR showed that molecular methods are more sensitive than the conventional methods and have the additional advantage of being rapid (Taylor et al. 2001; Thoen et al. 2006). As for IGRA, it has high reliability and reproducibility, minimizes direct handling of samples cultures, has no cross reactivity with BCG, and does not have a boost effect because it is an *in vitro* assay (unlike TST) (Bianchi et al. 2009). IGRA also has some of the same advantages of molecular methods.

In the case of examining farm animals for bovine TB, IGRA has various advantages including increased sensitivity and objective test procedures. More importantly, the test eliminates the need for a second visit to the farm and only requires 1-2 days, making it more rapid than culture and TST. However, IGRA also has drawbacks: it cannot distinguish between active and latent TB, it cannot be used for extrapulmonary TB diagnosis, it needs laboratory infrastructure, and its cost is high.

Comparing different detection methods, ZN smear had the highest specificity (100%) but the lowest sensitivity (74%). This may be due to its inability to detect less than 5,000-10,000 AFB per mL of specimen (de Waard & Robledo 2007). Therefore, if only ZN smear is used, TB patients may not be diagnosed, resulting in more complications for the patient and transmission of disease in the community (de Kantor et al. 1998). In addition, ZN smear cannot distinguish between MTB and other mycobacterial species (de Waard & Robledo 2007), eliminating its use as a sole method of detection.

The culture method has a higher specificity than real-time PCR and IGRA (99%) but lower sensitivity (84%). False negative results obtained by culture in 8/50 TB cases (16%) may be due to LJ culture contamination resulting in culture loss (de kantor et al. 1998).

Real-time PCR has a sensitivity of 92% and specificity of 97%. Real-time PCR was negative in 4/50 TB cases (8%); this might be due to the uneven distribution of bacilli in the sample, bad sampling, or suboptimal DNA extraction (Tortoli & Palomino 2007). In non-TB patients, real-time PCR was positive in 3/100 cases (3%); this false positivity may be due to laboratory contamination (Ani et al. 2009).

IGRA has the highest sensitivity at 96%, and a specificity of 89%. False negative IGRA results were obtained in 2/50 TB patients (4%). One of them was positive by real-time PCR, indicating that the patient might have weak T-cell response due to severe disease or might have been immunocompromised (Ravn et al. 2005). The other false negative sample was negative by real-time PCR as well and might have been sub-optimally processed or stored. The IGRA positive results in 11/100 non-TB cases may indicate LTBI in these patients. Thus, patients with positive IGRA results need follow-up to avoid developing active TB. These 11 samples were not detected by ZN smear or real-time PCR, and 10 out of the 11 samples were not detected by culture. This might indicate poor quality of respiratory specimens, as reported by the WHO (de Kantor et al. 1998).

Conclusion

IGRA is the method of choice for TB detection, as it was able to provide an accurate estimate of current TB infection rates among humans and animals in the examined community. IGRA can replace conventional methods for detection of TB, especially in countries like Egypt where BCG vaccination is a national policy. It can be used for the detection of TB in farm animals as well. It has the advantage of being easy, rapid, precise, highly sensitive and convenient.

Acknowledgements

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