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Review Article

Mycobacteriophage: Present and Future Possibility as a Therapy for Tuberculosis

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Abstract

Mycobacteriophages, first discovered around 72 years ago are viruses that infect mycobacterium species. Around 10427 Mycobacteriophages have been isolated till date, out of which, 1670 mycobacteriophage genomes have been sequenced. During 1960s and 1970s, phages created a big contribution to our knowledge of mycobacteria and following the development of typing techniques which were widely used in epidemiological studies of tuberculosis. After the invention of many antibiotics the study of phages got little attention. During past decade phages have attracted everyone's attention towards it since there has been a tremendous increase in antibiotic resistance leading to millions of death worldwide due to tuberculosis caused by Mycobacterium tuberculosis.

The incidence and mortality rate due to tuberculosis has stimulated the urge to explore more research aspects in mycobacteriophage research. Today their potential as diagnostic reagents is also being realized with the development of exciting advanced techniques for rapid bacterial detection and drug susceptibility testing, studying phage biology and as tools in recombinant DNA technology, thus facilitating the investigation of mycobacterial pathogenesis.

This review outlines the current scenario of tuberculosis as world's burden and role of phages in the control of tuberculosis by developing rapid diagnostic techniques and therapy for tuberculosis, which would be more cost effective and could reduce the duration of treatment. Phage-based diagnostic tests are at an early stage of their development; in future year's further technical innovation might improve their sensitivity, speed or convenience.

Keywords: Mycobacteriophage; Tuberculosis; Therapy; Diagnosis

Introduction

Presently, Mycobacteria is a major causes of morbidity and mortality in the worldwide. In India, as per the Global TB report of 2017 the estimated cases of TB were approximately 2,800,000 accounting for about a quarter of the world's TB cases (WHO Global TB report 2018). Mycobacterium tuberculosis inherits diverse intelligent strategies to survive inside the antagonistic environment of host cells. The global TB prevalence has become worse in recent years due to the emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains, as well as co-infection with HIV. Although Bacillus Calmette-Guérin (BCG) vaccine has nearly been used for a century in many countries, it does not protect adult pulmonary tuberculosis [1]. The hike in the multi-drug resistant tuberculosis worldwide has forced to search out newer ways to tackle this deadliest disease.

At present, TB control faces variety of challenges, including low sensitivity, poor specificity and treatment complications, long detection cycles of traditional diagnostic techniques and a decline in the immune function of traditional vaccines, including Bacillus Calmette Guerin [2]. Mycobacteriophage, the phage that infect mycobacteria, were first isolated in 1946 from samples of soil and leaf mould [3]. They were lytic phage which is able to infect fast-growing saprophytic mycobacteria such as Mycobacterium smegmatis. Slow-growing pathogenic bacilli of the Mycobacterium tuberculosis complex would get infected by mycobacteriophage were discovered [4]. As the number of mycobacteriophages grew a varied host range was revealed. One, mycobacteriophage DS6A [5], was found to be specific for bacteria of the Mycobacterium tuberculosis complex whilst others could be used to differentiate strains and were used in typing studies. Initially they were used to differentiate strains of clinical significance and schemes were established to type Mycobacterium avium, Mycobacterium kansasii, Mycobacterium xenopi, Mycobacterium fortuitum and M. tuberculosis [6]. Since then 10427 mycobacteriophages have been isolated and 1670 mycobacteriophage genomes have been sequenced [7]. As a member of the bacteriophage family, which are DNA viruses, mycobacteriophages are able to infect the host Mycobacteria specifically. L5 [8], D29 [9] and TM4 [10] are the mycobacteriophages, were the earliest to undergo genome sequencing and are the most widely used in the investigation of tuberculosis.

For diagnostic applications considerable progress has been made in the development of tests for pulmonary tuberculosis and in the detection of drug resistant disease. The phage-based tests are not as rapid as other alternative new technologies such as nucleic acid amplification. There is no need of investment in subtle instrumentation could prove enticing for poorly resourced laboratories wherever labour prices are low.

Meanwhile rigorous evaluation studies are needed on the accuracy of those tests currently available in order to determine their

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role in the control of mycobacterial diseases. Investigation of their robustness, costs and labour requirements will be required before the true benefits of the technology can be judged.

Present scenario

Mycobacterium tuberculosis has an extremely slow growth rate (24-hour doubling time), so phage typing can accelerate diagnosis by several weeks. This general concept of utilizing the relative rapid proliferation of mycobacteriophages has been a common subject of research for their subsequent development [11,12]. Work with mycobacteriophage as diagnostic or therapeutic tools has to this point concerned a little variety of bacteriophage with most studies concentrating on DS-6A, D29 and TM4 [13,14].The high specificity of DS-6A makes it an attractive candidate for anti-tuberculosis medical aid whereas D29 and TM4 are employed in tests for diagnosing and drug susceptibility. They both have a wide-host range and can be conveniently propagated in Mycobacterium smegmatis, fast-growing saprophytic mycobacteria [15]. They readily infect bacilli of the Mycobacterium tuberculosis complex including Mycobacterium bovis while TM4 also infects mycobacteria of the Mycobacterium avium complex [16]. The complete and the latest list of sequenced phages and their current status is available at the Phages DB (http://phagesdb.org) and Phamerator (http://phamerator.org) databases [17].

The success of bacteriophage as therapeutic or diagnostic agents will depend on their ability to infect the target bacilli, and the efficiency of infection is an important consideration in the design of such tools. Unfortunately, the factors affecting bacteriophage infection of mycobacteria are poorly understood and await further study. The means of attachment of the phage to the cell wall are not known. It has been speculated that the receptors used by D29 may be essential components of the cell wall. If a bacillus is simultaneously infected by large numbers of phage cell death may occur prior to phage replication [18,19]. Productive infection rates are highest in actively growing bacteria. David et al. (1980a) observed improved infection rates in actively growing Mycobacterium tuberculosis cells after washing and re-suspension in fresh broth. They speculated that a build up of lipids at the cell surface might be responsible for inhibiting phage binding but did not present direct evidence to support this. Other groups report improved infection rates when using bacteria grown as a film at the surface of liquid cultures [15]. Clumping of bacteria deters infection while detergents that might prevent aggregation such as Tween prevent attachment to the cell wall [20]. The kinetics of the phage infection cycle is related to host generation times and is extended in the slow-growing mycobacteria. DNA synthesis can be observed 2–4 minutes after infection of fast-growing Mycobacterium smegmatis but in Mycobacterium tuberculosis it is delayed for 20 min [21]. Similarly, the time taken from infection to cell lysis (length of the latent period) has been reported as 30 and 35 minutes for Mycobacterium smegmatis but is 2–3 hour with Mycobacterium tuberculosis [19,22,23]. The number of progeny virions released through cell lysis varies; an average burst size of 120 has been reported for D29 infection in Mycobacterium tuberculosis [24]. However, there is evidence from electron microscopy and biochemical studies that phage may be released by mycobacterium before complete lysis and death of the host cell [25,26] demonstrated improved infection rates and increased burst sizes with selected mutants of mycobacteriophage L1, a lysogenic phage closely related to D29.

Mycobacteriophage therapy

An alternative approach would be to use phages prophylactically, to protect other individuals from infection when in close contact with an infected patient Therapeutic utility is likely to require a cocktail of phages so as to minimize the incidence of phage resistance, just as antibiotics must be used in combination for TB treatment. However, the key property of phages in such a cocktail is that resistance to one phage does not confer resistance to the other phages. Unfortunately, very little is known mechanisms of resistance and this is an area that needs further investigation. The application of bacteriophage to treat disease is not a new idea and their application to nonmycobacterial diseases has been extensively reviewed elsewhere [27,28]. Phage therapy is currently used in Eastern Europe and countries of the former Soviet Union where it has been applied to infections of Staphylococcus, Pseudomonas, Escherichia, Klebsiella and Salmonella [29]. The mycobacteriophage have previously received less attention. However, the resurgence of mycobacterial disease has prompted fresh appraisal of their potential as therapeutic tools. Mycobacterial infections are difficult to treat, they are naturally resistant to many antibiotics and require multiple drug therapy for extended periods of time [30,31]. The minimum treatment for tuberculosis disease is 6 months; 2 months with four drugs, followed by 4 months of two drugs [32,33]. Failure to maintain adequate therapy may result in the development of drug resistance. Strains of multi-drug resistant tuberculosis (MDR-TB) that cannot be cured by standard treatment regimes are a serious threat to control the disease [34]. For Buruli ulcer the preferred treatment for severe cases is surgery as drug therapy may be ineffective because of the failure of antibiotics to penetrate the site of infection [35].

In the agricultural sector Mycobacterium bovis or MAP disease is not treated and control of these diseases is by the culling of infected animals [36,37]. The difficulty of treating mycobacterial disease and the emergence of drug resistance has encouraged scientists to investigate whether mycobacteriophage could provide a complementary means of therapy. Early attempts to treat laboratory animals infected with Mycobacterium bovis BCG and Mycobacterium tuberculosis were not successful [38,39]. It has been speculated that the treated animals may have been affected by toxic shock resulting from the lysis of large numbers of bacteria. More encouraging results were obtained by [40].who reported a reduction in lesions in the spleen, lungs and livers of guinea pigs following therapy with DS-6A. Similarly, more recent work demonstrated that phage therapy could have a beneficial effect in guinea pigs with disseminated tuberculosis [41]. However, the curative action was observed to be less than that of isoniazid monotherapy. Interestingly, the treatment with mycobacteriophage resulted in changed pathology, with decreased granuloma formation. Similar changes were observed by [38]. who also noticed an increase in features associated with sarcoidosis following simultaneous inoculation of guinea pigs with Mycobacterium tuberculosis and DS-6A. Killing intracellular pathogens such as Mycobacterium tuberculosis presents a tough challenge as in order to infect the target bacilli the phage need to transverse the mammalian cell membrane and survive in adverse intra-cellular environments such as reduced pH. Novel phage delivery systems are required and a possible strategy

has recently been identified where bacteriophage are transported into macrophages via non virulent carrier bacteria [42]. Macrophage cell lines infected with Mycobacterium tuberculosis or Mycobacterium avium were treated by the addition of Mycobacterium smegmatis infected with mycobacteriophage TM4. Following ingestion and destruction of the Mycobacterium smegmatis bacilli TM4 phages were released within the macrophage. A significant reduction in infection was observed in both the Mycobacterium tuberculosis and Mycobacterium avium experiments suggesting that TM4 bacteriophage had successfully infected and destroyed pathogenic bacteria within the macrophage. A second challenge to successful phage therapy is the presence of granulomas which might prove impenetrable to the bacteriophage. These are often observed in mycobacterial diseases and might prevent complete clearance of the bacteria [43]. Phage therapy might be more readily applicable to less visceral mycobacterial diseases such as Mycobacterium marinum or Buruli ulcer where the site of infection is accessible. However, no such studies have so far been reported. Further research is needed to overcome the existing technical barriers and enable the development of effective therapeutic tools. The emergence of drug resistant disease as a serious public health problem has sparked considerable scientific and public interest in this area of research (http://www.phagetherapy.com/ptlinks.html) but a role for mycobacteriophage in the treatment of tuberculosis and other mycobacterial infections has yet to be established.

Non-tuberculosis mycobacterium (NTM) is also a source of infection and may also represent possible targets for phage therapy. Attractive targets are Mycobacterium abscessus infections associated with cystic fibrosis (CF), which is often highly refractory to antibiotic therapies. Relatively little is known about the phage susceptibility profiles of M. abscessus clinical isolates, but a substantial proportion of sequenced M. abscessus strains carry one or more prophage [44,45] which could code for viral defense systems.

Diagnosis technique

One of the most commonly used mycobacteriophage for diagnostic tests is D29. First isolated from soil, it is a robust phage with a wide-host range that is easily maintained in the laboratory [4,46]. The ability of D29 to form visible plaques in a lawn of Mycobacterium smegmatis on overnight incubation enables rapid detection of plaque forming units (PFU) and Mycobacterium smegmatis may be used as an universal indicator strain to detect D29 propagated in slow-growing strains such a Mycobacterium tuberculosis or Mycobacterium Ulcerans Viral replication and production of progeny phage will only proceed in the presence of viable host bacteria and an increase in PFU indicates the presence of live mycobacteria. D29 was previously used to infect Mycobacterium tuberculosis bacilli extracted from animal tissues [23]. High multiplicities of infection are required to ensure good rates of infection [19] and when detecting small numbers of progeny phage it is necessary to differentiate between them and the excess inoculate remaining in the culture broth. To achieve this it is necessary to remove extracellular bacteriophage from the culture media during the latent phase of the infection. Extra-cellular phage inactivation was previously performed by dilution into a solution of anti-phage antibodies which bind and neutralize the phage particles [47]. However, the requirement for specialist antibodies and technical difficulties in ensuring complete inactivation of free viruses result in this technique being unsuitable for routine use. An alternative approach is differential chemical inactivation where an agent is used to kill those phage free in solution whilst not harming the host bacteria or any phage replicating within them. For mycobacteriophage this was previously achieved with sodium hydroxide [48] or sulphuric acid [49]. More recently, highly sensitive methods of detecting mycobacteriophage have been developed using iron II (ferrous) salts which inactivates D29 and related mycobacteriophage [50]. Samples containing <10 CFU of mycobacteria may be detected, a sensitivity approaching that of PCR and a hundred times greater than that achieved by microscopy [51].

The phage replication test by which samples are inoculated with phage, treated to inactivate extra-cellular phage and the progeny phage produced detected by plating in a lawn of Mycobacterium smegmatis provides a simple, low-cost method of detecting mycobacteria. The test provides a result in 3 days which is considerably faster than traditional culture methods but slower than microscopy or molecular amplification techniques where results may be obtained within 1 day. D29 has been used to detect M. tuberculosis in clinical specimens and a low-cost diagnostic test for pulmonary tuberculosis has been developed [46]. A commercial kit, FASTPlaqueTB (Biotec Laboratories Ltd, Ipswich, UK) has also been developed which is reported to use D29 [51]. Initial results obtained with this technology indicate that the sensitivity for detection of viable bacilli is much reduced when testing clinical specimens. Despite detection of tiny numbers of mycobacteria from in vitro cultures when applied to diagnosis of tuberculosis the phage test is not as sensitive as traditional culture methods. Results with the commercial test have been mixed, whilst not as sensitive as culture, the manufacturers have suggested it may have a role in diagnosis of microscopy negative specimens [52]. The manufacturers working in South Africa reported higher sensitivity than microscopy [51] while in an independent evaluation in Spain the test performed less well [53]. One study in Pakistan encountered serious problems of contamination which they partially solved by the addition of a cocktail of antimicrobials, reducing their sample loss to 12% [51].

Further, independent trials are needed to evaluate this technology to include assessment of economic and logistical factors. It is likely that improved sensitivity will be required before bacteriophage tests are judged suitable for routine diagnostic screening. Whilst culture remains the gold standard molecular amplification kits currently offer rapid testing with substantially higher sensitivities than microscopy. In a review of the literature Pfyffer found detection rates in smear negative respiratory specimens of between 36 and 92% [54]. Smear microscopy is a rapid and cheap test and the current practice is to examine three sputum samples per patient [55]. It may be preferable in poor resource settings where culture facilities are not available to improve the quality of microscopy rather than invest in new technology. The efficiency of bacteriophage infection is dependent on the physiological state of the target bacilli and the sensitivity of the phage tests is likely to be affected by the quality of the samples and how they have been stored. The reagents used to prepare sputum samples for culture are detrimental to phage infection and it has been suggested that a less stringent method of sample preparation might assist phage replication tests [56]. However, the need for rigorous decontamination when dealing with clinical specimens was

illustrated in the study in Pakistan [50]. It should be noted that the wide-host range of mycobacteriophage D29 means that these tests will also detect other mycobacterial infections and confirmatory tests may be required [53].

Techniques for Detecting Drug Resistance

The ability of bacteriophage to demonstrate susceptibility of mycobacteria to antibiotics has been recognised for many years. However, it is only within the last decade that serious efforts have been made to develop tests for routine drug resistance screening. In 1965, Tokunaga and Sellers investigated the effect of streptomycin on mycobacteriophage. They demonstrated that it would block phage replication in susceptible strains of Mycobacterium smegmatis but that replication continued in drug-resistant bacilli [57]. They also reported that at high concentrations it induced premature lysis of infected bacteria. Other workers reported similar effects with kanamycin [58]. Rifampicin, one of the major anti-mycobacterial drugs, is an inhibitor of nucleic acid synthesis and so prevents all bacteriophage replication in susceptible strains [59]. However, when the effect of ethambutol was examined it was found to have a partial effect [60]. Although the number of bacilli supporting phage replication decreased synthesis continued in a proportion of the population. This effect was thought to be caused by the indirect mode of action of this drug which is believed to inhibit synthesis of the mycobacterial cell wall. In 1980, David and colleagues investigated the inhibitory effects of clofazimine, colistin, rifampicin, streptomycin, dapsone, isoniazid and ethambutol on replication of mycobacteriophage D29 [23]. As a result of their investigations they concluded that phage could be successfully used to screen for anti-bacterial agents and that they might be useful when testing difficult to grow mycobacteria. However, unfortunately their attempts to infect Mycobacterium leprae were not successful [21].

Several tests for drug resistance have been developed using mycobacteriophage D29. The PhaB assay [61] compares the number of PFU produced with and without drug treatment, if the number of plaques from drug treated samples exceeds 1% of those from the untreated sample then the strain is classed as resistant. The test takes 4 d to perform and has been used for determination of susceptibility to the key anti-tuberculosis drug rifampicin [61,62]. One study sucessfully reduced the time of exposure to rifampicin to obtain results on the third day [63]. The test was also applied to isoniazid, ethambutol, streptomycin, pyrazinamide and ciprofloxacin but with the exception of streptomycin the method did not perform well and the authors found that despite using increased concentrations of drugs it was necessary to raise the proportion of plaques indicating a resistant strain from 1 to 10% [64]. A simpler rapid test based on a 96-well microplate format has been developed for high throughput screening of isolates for resistance to rifampicin. The test takes 48 h and has also been used to screen for streptomycin resistance [65]. A commercial test FAST Plaque TBRif (Biotec Laboratories Ltd) is marketed for screening cultures of Mycobacterium tuberculosis for resistance to rifampicin. The test is in a single-tube format and takes 48 h to perform. Although only small numbers of strains have been tested so far the results look very promising. In 10 of the 11 studies, reviewed 100% of the resistant strains were detected. However, some false positives were observed where susceptible strains were wrongly classified as resistant. The incidence of rifampicin resistance is low in most countries and high specificity will be required if these tests are to be used for routine screening. These studies indicate that phage replication technology offers an exciting prospect for rapid screening for drug resistance. Work is being undertaken in a number of laboratories to combine the diagnostic tests with drug susceptibility screening which would allow direct testing of sputum samples. Whether phage technology can deliver results comparable with those already obtained with molecular tests remains to be seen but that they can provide more rapid testing than conventional methods has already been demonstrated. When testing the FASTPlaque direct RMP test (Biotec Laboratories Ltd) the manufacturers correctly identified 11 of 15 rifampicin resistant and 134 of 154 susceptible tuberculosis cases in 48 h [66] while a study of 40 sputum specimens in Pakistan found a sensitivity for rifampicin resistance of 86% with a specificity of 73% [67]. Further studies are needed but phage technology may have advantages of cost and convenience over molecular technologies and so be more applicable to TB control in settings where resources are limited.

Luciferase reporter phage

An exciting new diagnostic tool was revealed in 1993 when Jacobs and colleagues reported the construction of a phage which incorporated the gene for luciferase, an enzyme normally found in fireflies. This recombinant phage has the ability to express the luciferase gene whilst infecting a mycobacterium. In the presence of luciferin substrate infected bacteria emit light that can be detected with a luminometer or by photosensitive film. The first luciferase reporter phage (LRP) was based on TM4, a lytic phage and subsequently reporter phage based on D29 and the temperate L5 have been constructed [68,69]. These LRP offer an elegant means of detecting viable mycobacteria and provide a rapid tool for drug susceptibility screening. However, reports of their application to clinical samples have been limited so far. In studies performed in Mexico using LRP for detection of mycobacteria in sputum 76% (54 of 71) of culture-positive specimens were detected [70]. This was less sensitive than microscopy which detected 93% (66 of 71) of the cases. A luminometer was used to detect the emitted light the positive LRP results were detected within 7 days. The phage used as LRP are not specific for Mycobacterium tuberculosis and they will also produce light when infecting other mycobacteria. A confirmatory test has been incorporated into the assay by using p-nitro-a-acetylamino-b-hydroxy propiophenone (NAP) which is inhibitory for Mycobacterium tuberculosis complex bacteria but not for other mycobacteria [71]. Following incubation with NAP and inoculation with LRP failure to produce luciferase confirms the strain is Mycobacterium tuberculosis while production of light indicates the presence of nontuberculous mycobacteria [72]. The method has been used to screen 53 isolates to confirm they were bacteria of Mycobacterium tuberculosis complex, 94% of the strains were confirmed by the LRP NAP method [70]. The LRP may also be used to screen for drug resistance and cultures of Mycobacterium tuberculosis may be tested against rifampicin within hours, whilst slow acting drugs such as ethambutol, isoniazid and ciprofloxacin can be tested in 2– 3 days [73]. Fifty Mycobacterium tuberculosis isolates were screened for susceptibility to four first-line antituberculosis drugs. The LRP detected resistance in three of three, two of two, six of seven and three of three of strains to rifampicin, streptomycin, isoniazide and ethambutol respectively. Complete

LRP results were obtained in 4 days but 94% of the results were available in 2 days, compared with a median turnaround time off for the routine culture test [70]. In a second study, LRP was used to identify Mycobacterium tuberculosis and screen 84 clinical isolates for drug resistance. The LRP NAP test had a sensitivity and specificity of 97 and 100%. The overall agreement for the drug susceptibility tests was 98.6%, four discrepant results were recorded where strains were found falsely resistant to ethambutol [74]**.** The LRP assay can be performed in 96-well plates and the emitted light detected by a luminometer, a system that lends itself to automation and thus the method would be convenient for large-scale screening programmes [73]. However, the high cost of such equipment would restrict its use in the poorly resourced laboratories of high-burden countries. Detection of emitted light by photosensitive film may offer a less expensive alternative, although the need to culture Mycobacterium tuberculosis whilst exposing to photographic film raises technical and safety considerations that require specialist equipment. Jacobs et al. working in the Bronx district of New York built a cassette that would hold a microwell plate over a sheet of sensitive photographic film. They named their device the "Bronx Box" and used it to screen isolates of Mycobacterium tuberculosis for resistance to rifampicin and isoniazid, obtaining results in 3 days [75-79]. This technology has been adapted by Sequella Inc. (Rockville, MD, USA) to a self-contained cassette using dental X-ray film. Evaluation studies are planned but no results are available at this time.

Conclusions

In past 72 years 10427 types of mycobacteriophages have been isolated and the genome sequences of 1670 types of mycobacteriophages have been completed. Mycobacteriophages have provided a wealth of insights into viral diversity and evolution, and played indispensable roles in the development of mycobacterial genetics. Mycobacteriophage genomes have several features, including diversity, a simple structure and amenability to genetic manipulation. Based on these characteristics, a shuttle plasmid was constructed for TB investigation using recombinant DNA technology. With improvements in genomics, shuttle plasmids have also been used to build different luciferase reporter phages and fluoromy¬cobacteriophages, which have contributed to the investigation of mycobacteria and TB. Following several years of limited studies, phage therapy is again an active area of investiga¬tion, particularly in bacteriophage lyase. As investigation into mycobacterial phages progresses, improvements in the current understanding of its role in tuberculosis, and particularly its diagnosis and treatment, is expected.

Studies of the application of mycobacteriophage to the treatment or diagnosis of mycobacterial infection have so far concentrated on human tuberculosis disease. For diagnostic applications considerable progress has been made in the development of tests for pulmonary tuberculosis and in the detection of drug resistant disease. The phage-based tests are not as rapid as other new technologies such as nucleic acid amplification. That they do not require investment in sophisticated equipment may prove attractive for poorly resourced laboratories where labour costs are low.

However, the requirement for microbiological safety facilities when isolating Mycobacterium tuberculosis may restrict their implementation to central reference laboratories in some developing countries. Phage-based diagnostic tests are at an early stage of their development; in future years further technical innovation may improve their sensitivity, speed or convenience. Meanwhile rigorous evaluation studies are needed on the accuracy of those tests currently available in order to determine their role in the control of mycobacterial diseases. Investigation of their robustness, costs and labour requirements will be required before the true benefits of the technology can be judged.

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