

Microbial Killing Activity of Peracetic Acid

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Abstract

In vitro killing activity of peracetic acid (Perasafe®) at a concentration of 0.26 per cent w/v was tested against *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi* A, *Acinetobacter baumannii*, *Sternotrophomonas maltophilia*, *Enterococcus faecium*, *Enterococcus faecalis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis* spore, *Mycobacterium tuberculosis* and human immuno-deficiency virus type I. Exposure to Peracetic acid (0.26% w/v) for 10 minutes resulted in massive killing of all the aforementioned organisms and spore.

Key word : Peracetic Acid, Bacteria, *Mycobacterium tuberculosis*, HIV-1

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Sterilization and disinfection of medical devices are critical components of health care delivery in order to protect patients from infections result-

ing from contaminated instruments and supplies. High temperature sterilization and high level disinfection are considered ideal. However, high tem-

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perature sterilization has limitations for temperature- or moisture-sensitive devices and supplies. Therefore, low temperature sterilization is required. Ethylene oxide has been the most widely used low-temperature sterilization compound. Health care facilities are currently searching for alternative compounds since ethylene oxide has several limitations. It has a potential toxic hazard to health care personnel and patients. It is a probable carcinogen and is flammable. Moreover, production of chlorofluorocarbons which are used as stabilizing agents in combination with ethylene oxide is prohibited by the Environmental Protection Agency. Alternatives for ethylene oxide include carbon dioxide, vaporized hydrogen peroxide, gas plasmas, ozone, peracetic acid and chlorine dioxide^(1,2).

The objective of the study was to determine the activity and stability of peracetic acid solution at a concentration of 0.26 per cent w/v against aerobic bacteria, bacterial spore, *Mycobacterium tuberculosis* and human immuno-deficiency virus type 1 (HIV-1).

MATERIAL AND METHOD

1. Bactericidal and Sporicidal Study

1.1. Test organisms

Bactericidal and sporicidal activities of peracetic acid were tested against a total of 15 clinical bacterial isolates, 2 reference strains of bacteria and 1 reference strain of sporulating bacterium. They included *E. coli* (2), *E. cloacae* (2), *K. pneumoniae* (2), *P. aeruginosa* (2), *S. typhi* (1), *S. paratyphi A* (1), *A. baumannii* (2), *S. maltophilia* (1), *E. faecium* (1), *E. faecalis* (1), methicillin-resistant *S. aureus* (MRSA) (2), and *B. subtilis* (1).

1.2. Reagents

The neutralizing dilution solution was tryptic soy broth containing 3 per cent Tween 80, 0.1 per cent histidine, 0.3 per cent lecithin and 0.5 per cent sodium thiosulphate. Peracetic acid solution was prepared by suspending 81 grams of peracetic acid (Perasafe®) powder in 5 liters (16.2 g/l) of tap water at 35°C and then left at room temperature.

1.3. Bactericidal & sporicidal activity test

Half a ml of a suspension of test organism containing $> 10^8$ cfu/ml was added to 4.5 ml of

peracetic acid solution. The mixture was gently mixed and left at room temperature. The mixtures were sampled at specific time intervals of 1, 5, 10 and 30 minutes after test organisms were inoculated by transferring 0.5 ml of test mixture into 4.5 ml of neutralizing dilution solution and mixing them vigorously. The test mixture was left for 10 minutes and then was spread on blood agar by calibration loop (0.01 ml). Viable counts were read after 48 hours of incubation at 37°C. Two types of control were included for each organism. Dilution of neutralization control which contained 4.5 ml of neutralizing dilution solution and 0.5 ml of 1:10 dilution of culture broth was done to test the influence of neutralizer alone on the test organism. To test the toxicity of the neutralizing solution to the test organism, neutralizer toxicity control was included. This control consisted of 4.5 ml of neutralizing dilution solution and 0.5 ml of peracetic acid solution which was added and mixed for 10 minutes before adding 0.5 ml of a suspension of the test organism. The test organism had to grow on both controls at the same concentration as in the culture broth alone.

1.4. Stability test

The stability of peracetic acid solution at room temperature was studied by testing the killing activity against four organisms; *E. coli*, *P. aeruginosa*, MRSA and *B. subtilis*, once a day until the activity decreased to the level of having growth of the test organism.

2. Virucidal Study

2.1 Test system

For a virucidal assay, human immunodeficiency virus type 1 (HIV-1, 100TCID₅₀, 100 µl) was exposed to an equal volume of peracetic acid (16.2 g/l, 100 µl) in a specific time at 1, 5, 10 and 30 minutes exposure. The residual of peracetic acid was removed immediately with neutralizer (8 ml) to prevent further action and reduce the cytotoxicity to cell culture for 1 hour. The culture media (1.8 ml) was added to give a final dilution by one-tenth. After that, ten fold dilutions of this mixture (50 µl) was plated in quadruplicate of 96 well-plate and incubated with C8166 cells (150 µl, 1×10^5 cells) at 37°C in 5 per cent CO₂ for 4-7 days. The residual virus was scored for a syncytium formation

on the target cells and measured for p24 antigen from a supernatant fluid. The effectiveness of the peracetic acid was considered as no syncytium formation on the indicator cells and in the absence of p24 antigen obtained from the residual HIV-1.

2.2 Control system

In order to demonstrate the cytotoxicity effects of test agents (non virucidal effect) to C8166 cells, a panel of controls was also included in quadruplicate. Peracetic acid control for the determination of the level of cytotoxicity of C8166 cells from peracetic acid were performed by incubating 10-fold dilutions of peracetic acid (50 μ l) with C8166 cells (150 μ l, 1×10^5 cells). Neutralizer control for the observation of the cytotoxicity effect from the neutralizer reagent was tested by incubating 10-fold dilutions of neutralizer (50 μ l) with C8166 cells (150 μ l, 1×10^5 cells). Adsorption control for investigation of the cytotoxicity from the residual peracetic acid after 1-hour inactivation with neutralizer was determined by mixing peracetic acid (100 μ l) and neutralizer reagents (8 ml) for 1 hour prior to the addition of the culture medium (1,900 μ l) to give the same dilution (1:10) as the test procedure. Then, the mixture (50 μ l) was added to the C8166 cells (150 μ l, 1×10^5 cells). Cell control for the observation of the morphology of cells by incubating the culture media (50 μ l) with C8166 cells (150 μ l, 1×10^5 cells). Virus control for the determination of virus infectivity by mixing 10-fold dilutions of virus stock (50 μ l) into C8166 cells (150 μ l, 1×10^5 cells). Finally, infectivity of the virus after adsorption by adding 10-fold dilutions of virus and peracetic/neutralizer mixture (50 μ l) after adsorption into C8166 cells (150 μ l, 1×10^5 cells). The presence of the syncytium formation on target cells and the presence of p24 antigen in the culture fluid were indicated as the infectivity of the stock virus.

2.3. Stability Test

In order to investigate the stability of peracetic acid, the working solution of peracetic acid was left at room temperature for up to 7 days. Virucidal effect was observed with 10 minutes exposure between the disinfectant and each batch of the same virus stock. The procedure was continued with the indicator cells and syncytium formation/p24 antigen was measured as described earlier.

2.4. Data analysis

The residual virus from the virucidal effect of peracetic acid and its stability were expressed as \log_{10} of the 50 per cent titration end point for infectivity (50% Tissue Culture Infectivity Dose, TCID₅₀) using the Spearman Karber program.

3. Anti-mycobacterial activity

3.1. Microorganisms preparation

Mycobacterium tuberculosis H37Rv cultured on Loewenstein-Jensen medium was transferred into Middlebrook 7H9 (M7H9) broth and the suspension of the organism was made. 100 μ l of cell suspension was transferred onto a Middlebrook 7H11 (M7H11) agar plate supplemented with OADC, spread and incubated at 37°C for 21 days. A single colony of *M. tuberculosis* from the M7H11 agar plate was collected into 100 ml of M7H9 broth and incubated at 37°C for 21 days. Ultrasonication of culture was performed for 10 minutes every 2 days of incubation to reduce the clump of cells. Glycerol was added to the culture for the final concentration of 10 per cent, mixed well and then 1 ml from each of the culture samples was aliquoted into 1.5 ml microcentrifuge tube and stored at -70°C until used.

3.2. Inoculum preparation

The stock inoculum from -70°C culture was thawed at room temperature, washed twice with M7H9 broth, spread onto the M7H11 agar plate and incubated at 37°C for 21 days. All colonies grown on the M7H11 agar plate were transferred into a screw-cap tube containing 5-6 glass beads (diameter = 5 mm). The tube was vortexed vigorously and 10 ml of sterile distilled water was then added. It was vortexed again. The big clumps of colonies were allowed to settle down for 30 minutes. The upper part of the suspension was transferred into a new tube and it was adjusted to achieve the turbidity to MacFarland No. 1 (approximately 3×10^7 cells/ml). This suspension was used for the preparation of the inoculum.

3.3. Peracetic acid preparation

81 grams of peracetic acid (Perasafe®) was dissolved with 5 liter of tap water at room temperature (final concentration = 0.26% w/v). The solution was vigorously stirred for 15 min.

3.4. Examination of disinfecting activity against *M. tuberculosis* H37Rv

100 µl of *M. tuberculosis* cell suspension was mixed with 900 µl of peracetic acid in a microcentrifuge tube and incubated at room temperature. At 1, 5, 10, 15 and 30 minutes of incubation, 10 µl of the mixture was taken to the tube containing the 990 µl mixture of Ringer and Tween neutralization-recovery medium to dilute and inactivate the peracetic acid. 100 µl of each dilution (at each time) was taken and spread onto the M7H11 agar plate (four sets of tests were made; set A-D and spread in duplicate for each set) and incubated at 37°C for 3 weeks. The growth of *M. tuberculosis* was examined and the number of colonies was counted. Sterile tap water was used instead of peracetic acid to serve as control. Tests were repeated using 24-h and 48-h prepared peracetic acid, respectively.

RESULTS

1. Bactericidal and Sporocidal Activity

Bactericidal and sporocidal activity and stability of peracetic acid are summarized in Table 1 and 2 respectively. Peracetic acid had a bactericidal and sporocidal effect on all 18 bacterial strains tested which covered a total of 12 species.

This activity was demonstrated even at a contact time of 1 minute for killing organisms at a high concentration of $\geq 10^9$ cfu/ml. The reduction in viability was $> 10^6$ cfu/ml. The solution at a concentration of 16.2 g/l under the stored condition of room temperature was stable and possessed bactericidal and sporocidal activity up to 5 days against all four bacterial strains tested ie. *E. coli*, *P. aeruginosa*, MRSA and *B. subtilis*. For killing activity against *E. coli*, the solution was stable up to 6 days.

2. Virucidal Activity

The availability of high-titre virus stock was $3 \log_{10}$ as scored by syncytium formation and $3.9 \log_{10}$ as measured by p24 antigen. For agent cytotoxicity on target cells, cytopathic effect was observed only in the disinfectant control at a dilution of 10^{-2} but not in any neutralizer or absorption controls compared to cell control as shown in Table 3. Thus, the set point for analysis of virus infectivity would be evaluated at a titre of 10^{-2} . Following exposure with the disinfectant, the residual infectious virus demonstrated as p24 antigen was not recoverable after 5-30 minutes contact as shown in Table 4. Although the exposure of virus and disinfectant were the equal volume at the starting point, this virucidal effect was finally equal at

Table 1. Bactericidal and sporocidal activity of peracetic acid.

organisms	Bacteria or spores remaining (cfu/ml)						Reduction in viability (cfu/ml)
	original	NT	NC	1 min	5 mins	10 mins	
<i>E. coli</i>	2.6×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>E. coli</i>	1.6×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>E. cloacae</i>	1.1×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>E. cloacae</i>	6.5×10^8	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>K. pneumoniae</i>	2.5×10^8	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>K. pneumoniae</i>	3×10^8	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>P. aeruginosa</i>	1.2×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>P. aeruginosa</i>	2.1×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>S. typhi</i>	1.6×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>S. paratyphi A</i>	1.1×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>A. baumannii</i>	1.6×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>A. baumannii</i>	2.1×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>S. maltophilia</i>	1.2×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>E. facium</i>	1.1×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>E. faecalis</i>	1.1×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
MRSA	2×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$
MRSA	6×10^8	$>10^6$	$>10^6$	0	0	0	$>10^6$
<i>B. subtilis</i>	2.5×10^9	$>10^6$	$>10^6$	0	0	0	$>10^6$

NT = Neutralizer toxicity control

NC = Dilution-neutralization control

Table 2. Stability of peracetic acid solution.

Organisms	Shelf life	Bacteria or spore remaining (cfu/ml)							
		Original	NT	NC	1 min	5 mins	10 mins	30 mins	60 mins
<i>E. coli</i>	Day 0	2.6×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 1	2×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 2	2×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 3	3.1×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 4	3.1×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 5	3.1×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 6	1×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 9	2×10^9	>10 ⁶	>10 ⁶	10	8	0	0	0
	Day 10	3×10^9	>10 ⁶	>10 ⁶	13	5	8	14	0
<i>P. aeruginosa</i>	Day 0	1.2×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 1	1.1×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 2	1.1×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 3	2.6×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 4	2.6×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 5	2.6×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 6	2×10^9	>10 ⁶	>10 ⁶	100	25	6	0	0
	Day 9	6×10^9	>10 ⁶	>10 ⁶	21	12	0	0	0
	Day 10	3×10^9	>10 ⁶	>10 ⁶	>10 ³	>10 ³	>10 ³	>10 ³	>10 ³
MRSA	Day 0	2×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 1	3×10^8	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 2	3×10^8	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 3	1.6×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 4	1.6×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 5	1.6×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 6	5×10^9	>10 ⁶	>10 ⁶	6	8	0	0	0
	Day 9	2×10^9	>10 ⁶	>10 ⁶	14	5	0	0	0
	Day 10	3×10^9	>10 ⁶	>10 ⁶	1000	30	24	20	0
<i>B. subtilis</i>	Day 0	2.5×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 1	6×10^8	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 2	6×10^8	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 3	1.5×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 4	1.5×10^9	>10 ⁶	>10 ⁶	0	0	0	0	0
	Day 5	1.5×10^9	>10 ⁶	>10 ⁶	0	0	30	36	0
	Day 6	1×10^9	>10 ⁶	>10 ⁶	12	100	30	36	0
	Day 9	3×10^8	>10 ⁶	>10 ⁶	3	3	4	3	0
	Day 10	3×10^8	>10 ⁶	>10 ⁶	4	3	12	0	0

a concentration half of the disinfectant at the period time of contact. When it was applied to the spillage solution, the concentration of the disinfectant was more diluted. Therefore, the application of the disinfectant at a concentration of 1:10 was investigated, the virucidal effect at 10 minutes exposure which is recommended by the company was sufficient to inactivate HIV-1 as well (Table 4). Finally, the virucidal effect of this disinfectant was not completely inactivated after preparation of more than 24 hours as the presence of p24 antigen (sensitivity as low as 1 ng/ml) but not with the syncytium formation by the naked eye as shown in Table 5.

3. Anti-mycobacterial Activity

Freshly prepared peracetic acid at the exposure time of 1 minute against *M. tuberculosis* showed several colonies of *M. tuberculosis* per plate. Whereas, the control, untreated *M. tuberculosis* cells using tap water instead of peracetic acid, showed a presence of more than 500 colonies/plate. Peracetic acid solution prepared for 24 hours had a low disinfecting activity since approximately 100 colonies/plate (1+) of *M. tuberculosis* were found. After 5 minutes of exposure, the freshly prepared peracetic acid as well as 24-hour prepared peracetic acid had good killing activity since no colony of *M. tuberculosis* was grown. Prepared peracetic

Table 3. Cytotoxicity of peracetic acid solution, neutralizer and disinfectant/neutralizer on C8166 cells.

Final dilution	Cytotoxicity control		
	Disinfectant	Neutralizer	Disinfectant/neutralizer
10 ⁻²	T	T (50%)	0
10 ⁻³	0	0	0
10 ⁻⁴	0	0	0
10 ⁻⁵	0	0	0

T = cytotoxicity presence

0 = no cytotoxicity presence

No cytopathic effect to cell control was observed.

Table 4. Effect of peracetic acid solution following a variety of exposure time to HIV-1_{MN} on C8166 cells.

Exposure time (minutes)	Residual infectious virus			
	Disinfectant : virus (1:1)		Disinfectant : virus (1:10)	
	Syncytium formation	p24 Ag	Syncytium formation	p24 Ag
1	0	10 ^{-2.5}	0	10 ⁻³
5	0	Neg	0	10 ⁻³
10	0	Neg	0	Neg
30	0	Neg	0	Neg

0 = No syncytium formation in C8166 cells

Neg = Negative for the presence of virus at a dilution of 10⁻² in supernatant fluid stock viral infectivity (3611 TCID₅₀) was 10⁻³ as scored by syncytium formation and 10^{-3.9} as measured by p24 antigen.

No cytopathic effect to cell control was observed

Table 5. Stability of peracetic acid solution against HIV-1_{MN} on C8166.

Stability of peracetic acid solution (days)	Residual infectious virus	
	Syncytium formation	p24 Ag
1	0	10 ^{-3.2}
2	0	10 ^{-2.9}
3	0	10 ^{-2.9}
4	0	Not tested
5	0	Not tested
6	0	Not tested

0 = No syncytium formation in C8166 cells

No cytopathic effect to cell control was observed

acid left at room temperature for 48 hours had minimal disinfecting activity against *M. tuberculosis*. There were 150 or more colonies/plate (3+) grown at 1 minute of exposure, 1+ at 5 minutes of exposure and none at 10 minutes of exposure as shown in Table 6.

DISCUSSION

The characteristics of an ideal low-temperature sterilant include high efficacy, rapid activity, strong penetrability, material compatibility, adaptability, ability to withstand an organic load, monitoring capability and cost-effective^(1,3). The results

Table 6. Activity of peracetic acid solution against *M. tuberculosis* H37Rv.

Set of experiment	Number of <i>M. tuberculosis</i> H37Rv colony after exposure time for				
	1 minute	5 minutes	10 minutes	15 minutes	30 minutes
Day 0					
A1	1+	0	0	0	0
A2	0	0	0	0	0
B1	1+	0	0	0	0
B2	0	0	0	0	0
C1	1+	0	0	0	0
C2	1+	0	0	0	0
D1	1+	0	0	0	0
D2	1+	0	0	0	0
Control 1			4+		
Control 2			4+		
Day 1					
A1	1+	0	0	0	0
A2	2+	0	0	0	0
B1	2+	0	0	0	0
B2	2+	0	0	0	0
C1	1+	0	0	0	0
C2	1+	0	0	0	0
D1	1+	0	0	0	0
D2	1+	0	0	0	0
Control 1			4+		
Control 2			4+		
Day 2					
A1	3+	0	0	0	0
A2	3+	1+	0	0	0
B1	3+	1+	0	0	0
B2	3+	1+	0	0	0
C1	3+	1+	0	0	0
C2	3+	1+	0	0	0
D1	3+	0	0	0	0
D2	3+	1+	0	0	0
Control 1			4+		
Control 2			4+		

0 = No growth; actual count = <50 colonies, 1+ = 50 -100 colonies; 2+ = 100-200 colonies; 3+ = 200-500 colonies; 4+ = > 500 colonies

from the present study indicated that peracetic acid is highly active against bacteria commonly caused hospital-acquired infections, bacterial spore, *M. tuberculosis* and HIV. This observation is in accordance with that from other studies^(4,5). However, the present study was only *in vitro* activity of peracetic against microorganisms without any instruments or devices and organic materials being taken into account. Studies conducted by an artificially contaminated endoscope with organisms also demonstrated efficacy of peracetic acid⁽⁶⁻⁸⁾. Peracetic acid at a concentration of 0.26 per cent w/v for 5 to 10 minutes is used for low temperature

sterilization in some hospitals in Europe⁽⁹⁾. The results from this study suggests that peracetic acid could be considered as an effective alternative for low-temperature sterilization. However, the issue of efficiency or economic analysis should be determined prior to adopting this alternative for routine practice for low-temperature sterilization in one's institution.

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ฤทธิ์ในการฆ่าเชื้อโรคของน้ำยากรดเพอร์อะซิติก

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ได้ศึกษาฤทธิ์ในการฆ่าเชื้อของน้ำยา peracetic acid (Perasafe®) ที่ขนาดความเข้มข้น 0.26% น้ำหนัก/ปริมาตรต่อเชื้อ *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi A*, *Acinetobacter baumannii*, *Sternotrophomonas maltophilia*, *Enterococcus faecium*, *Enterococcus faecalis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis* spore, *Mycobacterium tuberculosis* และ human immuno-deficiency virus type I พบว่าเมื่อเชื้อมีความเข้มข้นสูงและสปอร์ผสมกับ Peracetic acid นาน 10 นาทีจะตรวจไม่พบเชื้อมีชีวิตได้

คำสำคัญ : น้ำยาฆ่าเชื้อ, แบคทีเรีย, สปอร์, เชื้อวัณโรค, ไวรัสเอดส์, กรดเพอร์อะซิติก

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