Antistaphylococcal activity of Omani honey in combination with bovine milk

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Honey is well known for its antibacterial properties,[1–4] which act against a range of organisms including those that are highly resistant to antibiotics.[5,6] Honey has been used as an adjuvant to accelerate wound healing since ancient times and has been used sporadically in the treatment of burns.[2] The mild laxative, bactericidal, sedative and antiseptic characteristics of honey make it an important medicine.[7]

Bovine and human milk is reported to possess antimicrobial activities[8] and contains an array of bioactive substances including lysozyme, lactoferrin, immunoglobulins, growth factors and hormones, all of which are secreted in their active form by the mammary gland.[8,9] In combination with milk, honey provides excellent nutritional value and is recommended as the main source of nutrition in children.[10,11]

The antimicrobial activity of various Omani honeys has been investigated, and some possess excellent antibacterial activity against a wide spectrum of bacterial strains including *Staphylococcus aureus*.[12] In the present study, the extent to which Omani bovine milk inhibits the growth of *S. aureus* is assessed and whether or not a combination of honey and milk enhances antistaphylococcal activity.

An Omani honey sample (OH26), which previously demonstrated excellent activity against *S. aureus* (NCTC 6571), was compared to 29 other Omani honey samples after storage in sterile universal containers at room temperature (22–26°C) for no more than two months. Four different bovine milk samples (Al Marai, Al Rawabi, A'Safwa and Sohar) were obtained from farms immediately after pasteurisation and used directly. Honey and milk samples were diluted (1 in 2 to 1 in 16) using sterile distilled water.

A large *S. aureus* colony was emulsified in 0.1% peptone water (4 mL), yielding approximately 1 x 10^6 organisms, and used to swab diagnostic sensitivity test (DST, Oxoid, England) agar plates. Four wells (each 6 mm diameter) were cut in the DST agar plate and dilutions of honey or milk (50 µL) were added to each. Honey and milk samples were tested in triplicate. Plates were allowed to stand at room temperature for 10 min and then were incubated for 24 h at 37°C. Inhibition zone diameters were measured in millimetres and the average recorded.

Dilutions of honey and milk were made separately and in combination, using 2-mL volumes. A typical test included honey (diluted 1 in 2), milk (diluted 1 in 2) and a honey and milk combination (diluted 1 in 2). Tests were performed in triplicate and so a total of nine samples were prepared. To each sample was added 20 µL *S. aureus* (1 x 10^6 colony forming units [cfu]/mL, standardised with McFarlane turbidity tube 0.5) and mixed thoroughly. From these dilutions, a 10-µL volume was plated on blood agar and incubated for 24 h at 37°C. Subculture of each sample was repeated every two hours up to 24 h. The tests were run five times on five successive days. All colonial growth on the blood agar plates was counted and averaged for each set.

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Table 1. Zones of inhibition (mm) produced by various dilutions of Omani honey OH26 and four different milk samples.

	Concentration (v/v)				
	100%	50%	25%	12.5%	6.3%
Honey (OH26)	42	33	27	22	<10
Al-Marai Milk	20	11	00	00	00
Al-Rawabi milk	00	00	00	00	00
A'Safwa milk	00	00	00	00	00
Sohar milk	18	<10	00	00	00

Honey sample OH26, from the Al Batinah region (Al Kaborah), showed the highest level of *S. aureus* inhibition, and activity was evident up to a 1 in 8 dilution (12.5%). Table 1 shows the sizes of the zones of inhibition obtained with various dilutions of this honey sample and the four milk samples tested. Table 2 shows the percentage growth inhibition of *S. aureus* by honey, milk and a combination thereof at various time points.

Over the past decade, research into bioactive or biogenic substances derived from foods has been undertaken. Bioactive substances of food origin are considered to be dietary components that exert a regulatory activity beyond basic nutrition.

Although many studies of the antimicrobial activity of honey have been undertaken, it is believed that the present study is the first to address the antimicrobial effects of Omani honey in combination with bovine milk. The antistaphylococcal activity of Omani honey varies considerably,[12] and in this study it has been shown that not all milk samples possess antibacterial activity against *S. aureus* (Table 1). Two of the samples (Al-Rawabi and A'Safwa) not only failed to demonstrate antistaphylococcal activity but actually allowed significant growth of the organism. Combinations of honey and milk showed greater antistaphylococcal activity, enhancing the effect of honey alone by approximately 20%.

Honey is known to contain, phenol, fatty acids, lipids, amylases, ascorbic acid, peroxidases and fructose, and has a high osmolarity and low pH. These elements, acting alone or synergistically, may contribute significantly to the antibacterial activity of honey.[13–15] Although honey is known to have an antibacterial effect on different microorganisms, including those that are resistant to antibiotics,[5,6] its exact mode of action remains unclear.

Milk is generally accepted to have antimicrobial activities. Many bioactivities attributed to milk are encrypted in the primary structure of milk proteins and they require proteolysis for their effect to be realised. Proteolysis may release these biogenic peptides during gastrointestinal transit or during food processing.[8–9] These molecules include: opioid agonist and antagonist peptides; hypotensive peptides that inhibit angiotensin-I-converting enzyme (ACE); and mineral

Table 2. Percentage growth inhibition of *S. aureus* by honey, milk and a combination thereof at various time points.

	Time (hours)													
	0	1	2	4	6	8	10	12	14	16	18	20	22	24
Honey alone (50% v/v)	0	41	50	63	71	79	83	91	91	93	97	99	100	100
Al-Marai milk*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Honey and Al-Marai milk	0	45	66	59	91	93	89	96	98	100	100	100	100	100
Al-Rawabi milk*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Honey and Al-Rawabi milk	0	58	69	70	91	95	96	98	100	100	100	100	100	100

*Al-Rawabi and Al-Marai milk alone appeared to promote the growth of *S. aureus*.

binding, immunomodulatory, antibacterial, and antithrombotic peptides. Other antimicrobial fragments from bovine milk proteins are isracidin, casocidin-I and lactoferricin, which are released by pepsin digestion and have an effect against Gram-positive and Gram-negative bacteria.[8,9,16]

Although the exact mechanisms by which honey in combination with milk acts against *S. aureus* remains unclear, it is possible that honey releases some of the biogenic peptides in milk that have an antimicrobial effect. Clearly, this enhanced effect is important and is an area that requires more research.[17–19] Future work will involve repeat experiments using different strains of bacteria. □

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Detection and recovery of *Mycobacterium tuberculosis* from sputum specimens: comparison of the Mycobacteria Growth Indicator Tube and Löwenstein-Jensen medium

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There are no definitive guidelines available on the optimal use of the range of diagnostic tests (from simple acid-fast bacilli [AFB] microscopy to complex molecular biological techniques) that are now available to establish or rule out a diagnosis of tuberculosis. Isolation of mycobacteria from clinical samples by culture remains the cornerstone on

which definitive diagnosis of tuberculosis relies. However, culture on a solid medium, such as that of Löwenstein-Jensen (LJ), is both time-consuming (taking six to eight weeks) and insensitive.[1]

Preliminary studies evaluating the BBL Mycobacteria Growth Indicator Tube (MGIT) as a non-radiometric broth method for the growth and detection of AFB from clinical specimens have been reported.[2–4] The MGIT has been developed to circumvent some of the limitations described above, and contains modified Middlebrook 7H9 broth and a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with ruthenium pentahydrate). The fluorescence that indicates the presence of mycobaterial growth can be detected by transillumination using a 365-nm ultraviolet (UV) light.[5]

Combined use of a solid and liquid culture currently is the recommended gold-standard method for the detection of *M. tuberculosis* in countries where the disease is endemic.[6] The present study aims to compare *M. tuberculosis* recovery rates and the mean time required to detect the organism in clinical specimens using MGIT broth and LJ medium.

A total of 188 sputum specimens were obtained from patients suspected of having tuberculosis who attended four different hospitals (King Abdulaziz and King Faisal hospitals in Makkah, the Chest Diseases Hospital in Taif and the Tuberculosis Center in Jeddah) in the Western Province of Saudi Arabia. The study was carried out between December 2001 and May 2002.

All specimens were digested and decontaminated using the N-acetyl-cysteine–NaOH method described by Kent and Kubica.[7] After decontamination, smears were prepared from the concentrated sediments of the specimens for Ziehl-Neelsen (ZN) staining, and samples were inoculated in MGIT broth and on LJ medium.

The MGIT tube contains Middlebrook modified 7H9 broth (4 mL) with an oxygen-sensitive fluorescence sensor in the bottom of the tube to indicate microbial growth.[5] Prior to inoculation, oleic acid-albumin-dextrose-catalase (0.5 mL; BBL) and a mixture of antimicrobial agents (0.1 mL; PANTA, BBL) were added to each MGIT vial. Concentrated sediment (0.5 mL) added to the MGIT broth and 0.2 mL was inoculated on the LJ slant.

All inoculated media were incubated at 37 °C. The MGIT vials were examined daily with the 365-nm UV light for eight weeks. Vials that showed fluorescence comparable to the positive chemical control (0.4% sodium sulphite solution in an empty MGIT vial) and the positive growth control (quality control vial with *M. tuberculosis*) were considered positive. The MGIT vial was regarded as negative if no fluorescence was seen after eight weeks. Löwenstein-Jensen slants were examined weekly for eight weeks for visible appearance of colonies.

All positive samples were examined by ZN staining to confirm the presence of AFB. The MGIT vials with no fluorescence or colour formation but showing non-homogeneous turbidity and small grains or flakes in the broth were considered presumptive positives and were screened by ZN staining in the same way.

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Table 1. Smear and culture results for positive cultures.

Smear result	Positive cultures (%)	
	MGIT	LJ
Positive (*n*=8)	7 (87.5)	8 (100)
Negative (*n*=27)	9 (33.3)	11 (40.7)
Total (*n*=35)	16 (45.7)	19 (54.3)

Table 2. Recovery time for MGIT anf LJ culture methods.

	Detection time (days)		
	Minimum	Maximum	Mean
MGIT broth	5	33	21.5
LJ medium	19	48	35.5

Cultures positive for AFB were obtained from 35 (18.6%) specimens (Table 1). In three MGIT vials with no obvious fluorescence, positivity was suggested by the presence of non-homogeneous turbidity and small grains, and AFB positivity was confirmed by ZN staining. Recovery rates for *M. tuberculosis* were 16/35 (45.7%) with MGIT broth and 19/35 (54.3%) with LJ medium. A combination of methods recovered 31/35 (88.6%) isolates. Recovery times using the two culture methods are shown in Table 2.

Conventional methods for the identification of tuberculosis, such as AFB staining and cultivation of mycobacteria on solid media (LJ medium or Middlebrook agar), are relatively straightforward and well tested, but smears may lack sensitivity when used alone and culture may require several weeks' incubation for growth of mycobacteria to appear.[8]

Application of nucleic acid amplification (NAA) methods in mycobacteriology promises radical changes. However, the routine diagnostic use of these procedures has a number of drawbacks, including the need for specific quality assurance (QA) practices to prevent contamination and the high costs involved.[9] New techniques that use liquid media, such as the ESP Culture System (Difco Laboratories) or the BACTEC 460 TB radiometric system (Becton Dickinson), are able to detect mycobacteria in considerably less time,[10] but generally are labour intensive or have other limitations.

Clearly, desirable features of a diagnostic test for tuberculosis should include high sensitivity and specificity, speed, cost effectiveness, safety, simplicity and use an inefficient cultivation strategy. The MGIT method satisfies most of these criteria, being easy to handle, non-radiometric and not dependent on costly instrumentation.

The present study compared MGIT with the established LJ culture technique and defines the two most important parameters of a medium: the rate of recovery (sensitivity) and the mean time to detection (speed). Although the study design used was biased in favour of the MGIT system – larger inoculum size, results read more frequently – the differences were not statistically significant.

The false-negative rate observed with the MGIT system is somewhat surprising. This may have been due to the presence of only a small numbers of organisms, rather than a failure in the MGIT system. Furthermore, neither culture

system alone detected all the mycobacterial isolates, but yield increased when culture results from the MGIT broth and the LJ medium were combined. These findings support the recommendation that both solid and liquid media should be used in combination as a gold standard to achieve optimum results.[2–13]

The mean recovery time for *M. tuberculosis* in MGIT broth was shorter than that achieved with the LJ medium. This finding supports the work of Pfyffer[2] and other studies that report shorter mean times to isolation with the MGIT system.[3–14]

In conclusion, the speed with which mycobacteria are detected is the most obvious advantage of MGIT, which, together with the simplicity and flexibility of the system, makes it a suitable, non-radiometric alternative to other mycobacterial liquid media. Further study using a greater number and range of clinical specimens is required to establish the reliability of MGIT culture in the diagnosis of tuberculosis. In the meantime, the combination of MGIT broth and a solid medium such as LJ remains the gold standard for detection of mycobacteria. □

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Serial lipoprotein electrophoresis reveals the lipid changes in L-asparaginase-induced chylomicronaemia syndrome

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With the introduction of automated and direct assays of total cholesterol (TC), triglycerides (TG), high- and low-density lipoprotein cholesterol (HDL-C, LDL-C), the use of lipoprotein electrophoresis (LPE) has become less popular. However, in our experience, LPE remains a helpful and relatively simple tool that provides qualitative assessment of the nature of an underlying lipid disorder. In this brief study, a case of L-asparaginase-induced chylomicronaemia syndrome demonstrates the importance of LPE in revealing underlying lipid changes.

Since its discovery in 1953 by Kidd,[1] L-asparaginase has been incorporated in many combination chemotherapy protocols for the treatment of haematological malignancy, in particular acute lymphoblastic leukaemia (ALL) and non-Hodgkin's lymphoma. However, L-asparaginase has various side effects. Gastrointestinal disturbances, acute pancreatitis without hypertriglyceridaemia, haematological changes (thrombosis, leucopenia, bone marrow depression), nephrotoxicity, hepatotoxicity, somnolence, CNS agitation, convulsion and even coma have been reported.[2,3]

Disturbance of lipid metabolism is another complication not uncommonly seen.[4–6] Parsons *et al*.[5] reported that 67% of newly diagnosed children with ALL had fasting

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Table 1. Cumulative lipid results.

	Days after completion of asparaginase					
	Day 2	Day 3	Day 5	Day 6	Day 12	Reference range
Total cholesterol	6.4	13.8	14.3	11.1	5.8	<5.2 mmol/L
Triglycerides	30.0	60.6	11.1	12.5	1.1	<1.7 mmol/L
LDL-C (calculated)	–	N/A	N/A	N/A	4.0†	<3.4 mmol/L
LDL-C (direct)	–	Interfere*	1.6	1.5	–	<3.4 mmol/L
HDL-C	–	Interfere*	0.67	0.48	1.24	>1.0 mmol/L
Non-HDL-C‡	–	N/A	13.6	10.6	4.6	<4.2 mmol/L
Appearance	Lipaemic	Lipaemic	Turbid	Sl. turbid	Clear	
Apo B	–	1.14	–	–	1.01	0.49–1.15 g/L
Apo A1	–	0.48	–	–	1.18	1.10–1.80 g/L

*Direct assays for LDL-C and HDL-C on the Hitachi 912 analyser (Roche Diagnostics) would be interfered with when TG is greater than 13.6 and 10.0 mmol/L, respectively
†Indirect LDL-C is calculated by Friedewald equation provided that triglyceride is <4.5 mmol/L in fasting samples.
‡Non-HDL-C: Total cholesterol – HDL-C.

TG >2.6 mmol/L during L-asparaginase therapy. Severe hypertriglyceridaemia up to 103 mmol/L has been reported during combination treatment with corticosteroids and L-asparaginase, [6] serum TG >12 mmol/L is a major risk for acute pancreatitis, and a fatal case of acute necrotising pancreatitis three days after L-asparaginase treatment for leukaemia has been reported.[7]

It is postulated that L-asparaginase inhibits the activity of lipoprotein lipase (LPL) and is also associated with a decrease in apo CII concentration (the essential activator of LPL), as well as an increase in its inhibitor, apo CIII.[8] Moreover, it is suggested that decrease in the apo CII/CIII ratio in hypertriglyceridaemia may cause resistance to LPL and therefore be a part of the pathogenesis of hypertriglyceridaemia.[9,10] However, the detailed mechanism remains to be elucidated.

The patient discussed here is a 10-year-old boy with newly diagnosed T-cell ALL receiving L-asparaginase combination chemotherapy. The maintenance regimen included intravenous allopurinol, prednisolone, vincristine, daunorubicin, L-asparaginase, and intrathecal methotrexate and cytarabine. L-asparaginase was given at 5000 units/m^2 intravenously over one hour every three days for eight doses, starting on day 12 of the regime.

The patient's lipid profile was only checked two days after completion of treatment, when blood taken for routine blood tests appeared to be lipaemic. Measurements for TG and TC gave results of 30 mmol/L and 6.4 mmol/L, respectively. Common secondary causes of hyperlipidaemia (e.g., hypothyroidism, diabetes and obesity) were excluded, and he had no family history of lipid disorder. Plasma amylase was within the reference range. Subsequently, serial lipid profiles were performed to monitor the patient's progress (Table 1).

Lipoprotein electrophoresis was performed using a commercial system (Beckman Paragon; Fig. 1). Samples (5 µL) were run in barbitol buffer (pH 8.6) at 100 V for 45 min and the lipoprotein fractions were stained with Sudan Black B.

Triglyceride peaked at 60.6 mmol/L on day 3 after completion of L-asparaginase therapy and fell progressively and rapidly afterwards. Initial hypertriglyceridaemia

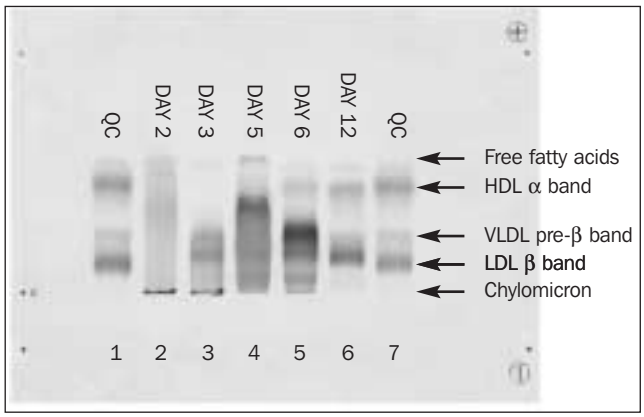

Fig. 1. Serial lipoprotein electrophoresis patterns. The smeared broad band between the β and pre-β bands are the lipoprotein remnants, which are most evident on lane 4 (day 5 post-asparaginase treatment, with TC: 14.3 mmol/L, non-HDL-C: 13.6 mmol/L and LDL-C: 1.6 mmol/L). QC: Normal control.

was attributed to the accumulation of chylomicrons, as indicated by the corresponding LPE, compatible with Fredrickson's World Health Organization classification of type I hyperlipidaemia or chylomicronaemia syndrome.

Chylomicrons contain >90% dietary triglycerides and some unesterified cholesterol. It is postulated that asparaginase, which has a plasma half-life of 4–15 hours,[11] inhibits LPL, resulting in decreased clearance of chylomicrons and resultant severe hypertriglyceridaemia.[8] In the case reported here, the lipid changes were transient and reversible, with the lipid profile returning to normal within two weeks. So, it would appear that LPL activity recovers gradually as the inhibitory effects of L-asparaginase wear off, and enzyme levels return to normal after the drug is discontinued.

Triglycerides in the chylomicrons are hydrolysed and cholesterol is taken up by HDL for esterification and transfer to other lipoprotein fractions (e.g., chylomicron remnants and intermediate-density lipoprotein particles).

This mechanism could explain the reciprocal increase of TC and non-HDL-C fractions that occurred concurrently with the fall in TG, and was demonstrated by serial changes in corresponding LPE pattern.

High-throughput automated assays for lipid testing are convenient to use and readily accessible. However, LPE retains a useful role in the investigation of lipid disorders by providing qualitative information that may be missed when samples are only analysed for TC, TG, HDL-C and LDL-C levels by an automated assay method. Here, underlying changes in lipid metabolism in a patient with L-asparaginase-induced chylomicronaemia syndrome were demonstrated, as was the value of serial LPE. □

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