SUPPLEMENT 7

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Research Fund for the Control of Infectious Diseases
Health and Health Services Research Fund

Research Dissemination Reports

控制傳染病研究基金
衞生及醫護研究基金

研究成果報告

Viral Hepatitis
病毒性肝炎

Human Immunodeficiency Virus
人類免疫缺陷病毒

Tuberculosis
結核病

Antibiotic Resistance
耐藥性

Supplement 7

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HONG KONG MEDICAL JOURNAL 香港醫學雜誌
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Research Fund for the Control of Infectious Diseases
Health and Health Services Research Fund

Research Dissemination Reports

ANTIBIOTIC RESISTANCE

Editorial

Dissemination reports are concise informative reports of health-related research supported by funds administered by the Food and Health Bureau, for example, the *Research Fund for the Control of Infectious Diseases and Health and Health Services Research Fund* (which were consolidated into the *Health and Medical Research Fund* in December 2011). In this edition, ten dissemination reports of projects related to viral hepatitis, human immunodeficiency virus (HIV), tuberculosis (TB), and antibiotic resistance are presented. In particular, three projects are highlighted due to their potentially significant findings, impact on healthcare delivery and practice, and/or contribution to health policy formulation in Hong Kong.

Chronic hepatitis B virus (HBV) infection occurs in 10% of the obstetric population in Hong Kong. There is concern that modulation of the mother's immune system as a result of pregnancy facilitates flare-up of chronic HBV infection and increase infectivity. Lao et al[1] determined the effect of pregnancy on the activity and replication of HBV during the three trimesters and identified the most sensitive clinically applicable markers of viral activity and maternal inflammation. They found HBV DNA in half of asymptomatic HBsAg-positive mothers. In-utero infection was associated with a maternal positive HBeAg status and higher HBV DNA load throughout pregnancy. The authors suggest that HBV DNA testing for every HBsAg carrier mother should be conducted, if feasible.

Coinfection of a host organism with hepatitis C virus (HCV) and HIV alters the course of infection of each virus. Yi at al[2] examined the gene expression profiles of CD8+ and CD4+ T cells in treatment-naïve mono- and co-infected individuals. A wide variety of gene pathways was found to be affected. The study findings offer new insight into disease progression in HIV/HCV co-infection, and may help to identify new markers for its management.

Tuberculosis is a highly infectious airborne disease. Healthcare workers are at increased risk of infection because of exposure to infectious patients. The tuberculin skin test has been used to diagnose latent tuberculosis infection (LTBI) with variable success, as the majority of local residents have been inoculated with Bacillus Calmette-Guerin (BCG) vaccine at birth. In-vitro interferon gamma release assays (IGRA) can identify individuals infected with TB who have been vaccinated with BCG. Tsang et al[3] determined the applicability of IGRA to daily use including contact investigation. The findings of this study have influenced the Hospital Authority guidelines on control of transmission of TB in healthcare settings and the behaviour and practice of research end-users, where an increase in utilisation of IGRA in investigation of LTBI, especially during outbreak/contacts investigation was observed.

We hope you will enjoy this selection of research dissemination reports. Electronic copies of these dissemination reports and the corresponding full reports can be downloaded individually from the Research Fund Secretariat website (http://www.fhb.gov.hk/grants). Researchers interested in the funds administered by the Food and Health Bureau also may visit the website for detailed information about application procedures.

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Effect of pregnancy on the activity and infectivity of hepatitis B virus in women with chronic hepatitis B infection

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KEY MESSAGES

1. One-half of hepatitis B surface antigen carrier mothers demonstrated hepatitis B virus (HBV) activity during pregnancy.
2. Hepatitis B e antigen (HBeAg) was the best maternal marker for HBV activity in pregnancy, but 59% of mothers with circulating HBV DNA were HBeAg negative.
3. In mothers with no detectable HBV DNA in the first trimester, viral activity still increased from 19.6% in the second trimester to 30.4% in the third trimester to 50% after delivery, but none of their infants had in-utero HBV infection.
4. In-utero HBV infection was evident in 8% of infants, and was related to maternal HBeAg status and high HBV DNA level during all three trimesters.

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Introduction

Chronic hepatitis B virus (HBV) infection, identified by a positive hepatitis B surface antigen (HBsAg) test, occurs in 10% of the obstetric population in Hong Kong.[1] The greatest concern is the high risk of vertical transmission,[2] because the suppression of the Th-1 and enhancement of the Th-2 immune systems during pregnancy may facilitate flare up of chronic HBV infection and increase infectivity. Antenatal screening with HBeAg detects only 10.5% of mothers with anti-HBe who have a viral load $>10^4$ IU/mL.[3] A detectable, and especially high level of, maternal serum HBV DNA is associated with a higher rate of intrauterine infection and consequent failure of passive-active immunoprophylaxis in the offspring.[4] Maternal antenatal antiviral treatment to prevent intrauterine infection can only reduce (not eliminate) the risk.[5]

Given the high prevalence of mothers with HBV infection in our community, routine assessment of every mother throughout pregnancy to monitor their viral DNA level enables selection of high-risk cases for treatment. Owing to resource and logistical concerns, the alternative is to screen and select high-risk women for antenatal treatment. Better understanding of the enhancement effect of pregnancy on HBV activity and infectivity, including the gestational effect, the magnitude of enhancement, and clinical and laboratory features of inflammation is necessary. We hypothesised that pregnancy is associated with increased viral activity in a significant proportion of women, some of whom will manifest features of inflammation that can be identified through antenatal monitoring. We aimed to (1) determine the effect of pregnancy on the activity and replication of HBV during the three trimesters, (2) identify the most sensitive clinically applicable markers of viral activity and maternal inflammation, and (3) examine the relationship of the effect of pregnancy and maternal HBV DNA level with cord blood HBV DNA positivity, taken as evidence of intrauterine infection.

Methods

This was a prospective longitudinal observational study conducted from October 2009 to March 2012 on asymptomatic pregnant women with positive antenatal HBsAg screening who had a singleton pregnancy and negative Down's screening.

As no data on HBV activity during pregnancy were available, we assumed this to be up to 20%. Assuming a type 1 error of 0.05 and a power of 80%, 65 to 174 women were required for the study. Although the study could tolerate a default rate of up to 15%, financial constraints limited the target number to 170 women screened positive for HBsAg.

Screening for maternal HBsAg was routinely performed at booking. Eligible subjects were recruited in the clinic and then invited to attend serial assessments at around 20-24 weeks gestation, 34-36 weeks gestation, and 6 weeks and 6 months after delivery. Blood samples were obtained for measurement of liver function, HBeAg status, HBV DNA level, complete blood count, and serum

ferritin and sensitive C-reactive protein (CRP). At delivery, umbilical cord blood was obtained for measurement of HBV DNA to determine whether in-utero transmission had occurred. HBV DNA measurements were performed using TaqMan real-time polymerase chain reaction (range of detection, 10^2 to 10^9 copies/mL; correlation coefficient of the standard curve, >0.990). The obstetricians were blinded to test results. Nonetheless, if the alanine transaminase (ALT) level was elevated, referral was made to the medical clinic for further assessment. Those who were on antiviral treatment before or during pregnancy were excluded. After delivery, newborns received passive (immunoglobulin) and active (vaccine) immunisation against hepatitis B as per protocol.

Not all recruited women attended all the antenatal or postnatal assessments, and a cord blood sample was not available in all cases, thus HBV DNA results at each time point varied. The final longitudinal study cohort comprised subjects who attended all three antenatal assessments, although not all had a complete set of cord blood or postnatal HBV DNA results.

Results

A total of 278 subjects were recruited; 32 of them were excluded for a variety of reasons: ten became HBsAg negative in the index pregnancy, three had abortions, four were twin pregnancies, five received antiviral treatment, and ten were lost to follow-up. For the remaining 246 subjects, their mean age was 32.1±4.3 years and mean body mass index was 22.0±3.1 kg/m^2; 41.5% were nulliparous, and 241 (98.0%) delivered at the Prince of Wales Hospital. Mean gestation at delivery was 38.5±3.2 weeks and mean birth weight was 3121±557 g. Regarding HBV activity, 27% of mothers were HBeAg positive, 42% to 61% of mothers had HBV DNA detected during pregnancy and postpartum, and 8% of infants had in-utero infection (Table 1).

In 157 (64%) subjects who attended all three antenatal assessments, HBV DNA was detected in 111 and not detected in 46. The two groups were comparable in terms of maternal characteristics, gestation at delivery, birth weight, and the incidence of obstetric complications, but the former had an elevated serum ALT level at all three assessments (P=0.006 to P<0.001) and elevated ferritin level in the third trimester (P=0.022) [Table 2].

According to the first assessment findings, positive HBeAg status identified maternal HBV viral activity (odds ratio=31.2, 95% confidence interval=4.14-234.32), but 59.1% of HBeAg negative mothers also had circulating HBV DNA (Table 3). Most women with HBV DNA detected subsequently had HBV DNA detected at the first assessment, whereas 19.6% (9/46) and 30.4% (14/46) of those without HBV DNA detected initially also had HBV DNA detected in the second and third assessments, respectively. Nonetheless, in-utero infection was confined to those with HBV DNA detected initially. After delivery, HBV DNA detection was similar to that during the second and third trimester (all above 80%), but, alarmingly, 46.5 to 51.2% of women in whom HBV DNA was not detected initially had HBV DNA detected at the postnatal visits, compared with 19.6% and 30.4% at the second and third antenatal assessment, respectively.

For infants with in-utero infection (16/127 or 13%), the incidence of positive maternal HBeAg status was significantly higher (93.8% vs 19.8%, P<0.001), and maternal HBV DNA level was also higher at the first assessment (8.23±1.20 vs 4.40±1.93 $\log_{10}$ copies/mL, P<0.001), second assessment

TABLE 1. Maternal condition and hepatitis B virus (HBV) activity

Parameter	No. (%) of subjects
Nulliparous women	102/246 (42)
Caesarean delivery	61/246 (25)
Hepatitis B e antigen positive	67/235 (27)
Elevated alanine transaminase level (≥55 IU/L) at 1st assessment	13/192 (5)
Maternal HBV DNA detected at 1st assessment	117/168 (48)
Maternal HBV DNA detected at 2nd assessment	139/205 (57)
Maternal HBV DNA detected at 3rd assessment	146/210 (59)
Maternal HBV DNA detected at delivery	102/165 (42)
Placental HBV DNA detected	64/180 (26)
Cord blood HBV DNA detected	20/173 (8)
Maternal HBV DNA detected at 6 weeks postpartum	149/195 (61)
Maternal HBV DNA detected at 6 months postpartum	135/183 (55)

TABLE 2. Maternal characteristics and pregnancy outcome in subjects with or without hepatitis B virus (HBV) DNA detected at the first assessment

Parameter	Mean±SD or No. (%) of subjects		P value
	HBV DNA detected (n=111)	HBV DNA not detected (n=46)	
Maternal age (years)	32.1±4.3	32.8±4.0	0.368
Weight (kg)	55.2±8.6	56.5±10.8	0.440
Height (cm)	159.1±5.3	158.0±5.7	0.242
Body mass index (kg/m^2)	21.8±3.1	22.6±3.9	0.154
Born in Hong Kong	57/110 (51.8)	14/46 (30.4)	0.014
Gravidity=1	24 (21.6)	10 (21.7)	0.987
Parity=0	41 (36.9)	15 (32.6)	0.606
1st assessment			
Haemoglobin (g/dL)	11.8±1.0	11.7±1.4	0.683
White cell count (x10^9/L)	8.3±1.8	8.3±1.9	0.824
Alanine transaminase (IU/L)	25.8±18.3	20.1±14.6	0.006
Ferritin (pmol/L)	157.0±134.3	168.6±145.4	0.713
C-reactive protein (mg/L)	2.9±2.6	4.8±6.7	0.427
2nd assessment			
Haemoglobin (g/dL)	11.2±0.8	11.3±0.8	0.646
White cell count (x10^9/L)	8.8±1.9	9.1±2.4	0.428
Alanine transaminase level (IU/L)	35.3±124.5	16.1±5.7	<0.001
Ferritin (pmol/L)	81.9±120.3	73.7±81.5	0.753
C-reactive protein (mg/L)	2.9±3.0	4.4±5.2	0.423
3rd assessment			
Haemoglobin (g/dL)	11.4±1.1	11.2±1.1	0.197
White cell count (x10^9/L)	8.2±2.1	8.0±2.2	0.543
Alanine transaminase level (IU/L)	22.9±20.2	15.0±5.2	<0.001
Ferritin (pmol/L)	43.2±31.5	36.5±42.1	0.022
C-reactive protein (mg/L)	3.2±3.6	3.5±3.4	0.565
Gestation at delivery (weeks)	39.0±1.3	39.2±1.2	0.315
Birthweight (g)	3162±386	3138±419	0.739

(8.13±1.16 vs 4.45±2.18 $\log_{10}$ copies/mL, P<0.001), and third assessment (7.93±1.30 vs 2.91±2.60 $\log_{10}$ copies/mL, P<0.001). There was no significant difference in infant characteristics, Apgar score, or cord blood pH and base deficit level.

Discussion

This study longitudinally examined HBV viral activity during pregnancy in asymptomatic HBsAg positive mothers. Although only 5% of women had an elevated ALT level at their initial assessment, HBV DNA was detected in half, and this proportion remained consistent across the three trimesters. Furthermore, one in 12 infants showed evidence of intrauterine HBV infection. A similar proportion of these mothers also showed evidence of HBV viral activity for up to 6 months postpartum. These women had persistent viral activity during and after pregnancy despite being asymptomatic.

Mothers with initial HBV DNA detection had a significantly higher incidence of HBeAg positivity (odds ratio=31.15) and elevated ALT level throughout pregnancy. Nonetheless, HBeAg status was unreliable in the identification of those with HBV activity: 59.1% of mothers with circulating HBV DNA were HBeAg negative. Similarly, the markedly overlapping ALT level, ferritin, CRP, and blood count were unhelpful. Therefore, HBV DNA testing for every HBsAg carrier mother is suggested if feasible. If not, at least HBeAg testing and serial ALT measurements should be performed.

The initial absence of HBV DNA could not exclude increased viral activity later on, as some of these subjects had HBV DNA detected in the second (19.6%) and third (30.4%) trimesters. The incidence

TABLE 3. Subsequent maternal hepatitis B virus (HBV) activity in relation to HBV DNA status at first assessment

Parameter	No. (%) of subjects		P value
	HBV DNA detected (n=111)	HBV DNA not detected (n=46)	
HBeAg positive status	45/110 (40.9)	1/46 (2.2)	<0.001
2nd assessment			<0.001
HBV DNA detected (n=106)	97/111 (87.4)	9/46 (19.6)	
HBV DNA not detected (n=51)	14/111 (12.6)	37/46 (80.4)	
3rd assessment			<0.001
HBV DNA detected (n=107)	93/111 (83.8)	14/46 (30.4)	
HBV DNA not detected (n=50)	18/111 (16.2)	32/46 (69.6)	
Cord blood HBV DNA status			0.006
Positive (n=16)	16/92 (17.4)	0/35 (0)	
Negative (n=111)	76/92 (82.6)	35/35 (100)	
6 weeks postnatal assessment			<0.001
HBV DNA detected (n=115)	93/104 (89.4)	22/43 (51.2)	
HBV DNA not detected (n=32)	11/104 (10.6)	21/43 (48.8)	
6 months postnatal assessment			<0.001
HBV DNA detected (n=104)	84/96 (87.5)	20/43 (46.5)	
HBV DNA not detected (n=35)	12/96 (12.5)	23/43 (53.5)	

increased to 51.2% at 6 weeks and 46.5% at 6 months postpartum. Although no in-utero infection was found in this subgroup, these subjects might have a higher maternal risk than those with HBV DNA detected initially. Further studies are required to elucidate the long-term maternal implications.

In-utero infection was associated with a maternal positive HBeAg status and higher HBV DNA load throughout pregnancy, but infant characteristics were comparable. This suggests maternal tolerance to chronic HBV infection with no consequent adverse perinatal outcome in most cases.

Conclusion

Half of the asymptomatic maternal carriers of HBsAg showed evidence of HBV activity, and subjects with undetectable HBV DNA in the first trimester had increased viral activity in the second (19.6%) and third (30.4%) trimesters and within 6 months of delivery (around 50%). No in-utero infection was found in mothers with initial undetectable viral activity. HBeAg was the best maternal parameter to identify subjects with viral activity; HBV DNA level should be measured for every HBsAg carrier mother, as 59% of HBeAg negative mothers also have circulating HBV DNA.

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COLD-PCR for early detection of hepatitis B virus antiviral drug resistance mutations

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KEY MESSAGES

1. Co-amplification at lower denaturation temperature-PCR (COLD-PCR) was developed for early detection of hepatitis B virus (HBV) drug resistance mutations.
2. With a simple alteration of denaturation temperature in the thermal cycle, COLD-PCR could detect drug resistance mutations that existed at a level of 5-10% within a mixed pool, compared with a level of ≥25% for conventional PCR.
3. In patients prescribed lamivudine or telbivudine, COLD-PCR was more sensitive than conventional PCR, with a higher mutation detection rate.
4. COLD-PCR is useful for patient monitoring, as it is more sensitive than conventional PCR in early detection of drug resistance mutations.

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Introduction

Nucleos(t)ide analogues (NAs) are effective therapeutic agents for the treatment of chronic hepatitis B virus (HBV) infection. However, long-term use of NAs is often hampered by the emergence of drug resistance mutations, causing potentially serious consequences such as liver decompensation and mortality. A more sensitive method for early detection of drug resistance mutations is needed.

Conventional PCR amplification of HBV DNA followed by direct sequencing of the purified amplicons for detection of drug resistance mutations has two advantages. First, it can detect any novel mutation within the amplicons. Second, it is relatively inexpensive. However, it cannot detect a low level of mutations that comprise <20% of the total viral population.

Co-amplification at lower denaturation temperature PCR (COLD-PCR) has been used for enrichment of a low level of variants within a mixed pool of sequences.[1] COLD-PCR relies on slight changes to the melting temperature (T_m) in the DNA sequence caused by mutations within the sequence. For each DNA sequence, there is a critical denaturation temperature (T_c) below which PCR efficiency decreases abruptly.[1] T_c is lower than T_m and is dependent on the DNA sequence itself. When the denaturation temperature of PCR is set to T_c (instead of the usual 94°C), DNA amplicons with different mutations will have different amplification efficiencies. This property enables selectively enrich low-level mutations in a mixed pool.

The principle for COLD-PCR is shown in Fig 1. Like conventional PCR, COLD-PCR starts with a denaturation step at 94°C. Following denaturation, an intermediate hybridisation temperature of 70°C is used to promote cross-hybridisation of mutant and wild-type alleles, forming a heteroduplex. Then, with a denaturation temperature at T_c, the heteroduplex is selectively denatured and subsequently amplified, whereas the wild-type homoduplex does not amplify efficiently. As a result, mutations that exist in a minority are enriched by COLD-PCR and detected by subsequent sequencing.

This study aimed to (1) develop a modified COLD-PCR method to detect common HBV drug resistance mutations in patients undergoing lamivudine or telbivudine therapy (two of the licensed NAs) and (2) compare the performance of COLD-PCR with that of the LiPA HBV drug resistance assay (Fujirebio Europe, Belgium) and conventional PCR.

Results

The execution of COLD-PCR depends on the experimental identification of T_c. Cloned wild-type HBV DNA and HBV DNA with representative drug resistance mutations were used as templates for PCR at different denaturation temperatures. Using a conventional denaturation temperature of 94°C, all wild-type and mutant sequences were amplified efficiently with Sybr-green-based real-time PCR, and experiments were performed with decreasing denaturation temperatures. PCR amplification was

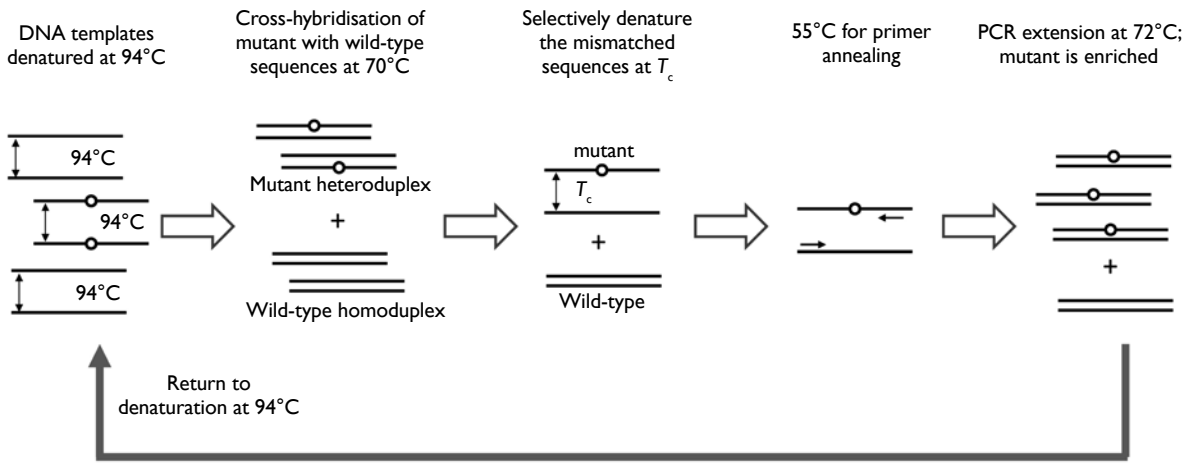

FIG. Principle of co-amplification at lower denaturation temperature PCR: mutation sites in the minority mutant template are shown as open circles (the figure is modified from a study by Li et al[1]; copyright permission to adapt that figure was obtained from Macmillan Publishers).

not observed when the denaturation temperature <78°C, which is defined as T_c when amplification efficiency decreases abruptly. Thus, the T_c for the PCR amplicon was determined to be 78°C.

Both COLD-PCR and conventional PCR were used to detect HBV mutations in mixtures of various proportions of cloned wild-type and mutant HBV DNA. Conventional PCR could detect drug resistance mutations only when the mutant plasmids existed at a level of ≥25% within the mixture, whereas COLD-PCR could detect drug resistance mutations at a level of 5-10% within the mixture.

The performance of the LiPA assay, COLD-PCR, and conventional PCR in detecting drug resistance mutations was compared in 106 patients treated with lamivudine and 30 patients treated with telbivudine. These patients have been followed up in our centre and previously reported to have virological breakthrough during follow-up.[2,3] Among these 136 patients, lamivudine/telbivudine-resistant mutations rtM204V/I were detected in 129 (95%), 108 (79%) and 84 (62%) patients by the LiPA assay, COLD-PCR, and conventional PCR, respectively. Drug resistance mutations were detectable by all three methods in 84 patients. To investigate whether these three methods can detect early drug resistance mutations, we attempted to detect rtM204V/I mutations from these 84 patients at 6-12 months before the previous mutation detection time points. In five samples taken from the earlier time point, HBV DNA was not amplifiable by PCR by any of the three methods. When analysing the samples taken from both time points collectively, COLD-PCR was also more sensitive than conventional PCR: 35 (16%) samples had rtM204V/I detected by COLD-PCR but not by conventional PCR, while all samples with mutations detected by conventional PCR were detected by COLD-PCR.

Discussion

Detection of drug resistance mutations is essential in the management of patients with antiviral therapy. Conventional PCR often cannot detect minority variants. In this study, COLD-PCR could detect HBV drug resistance mutations at a level of 5-10%, whereas conventional PCR could detect HBV mutations only at a level of ≥25%.

Among the three methods tested, the LiPA assay had the highest mutation detection rate in patients treated with lamivudine or telbivudine compared with COLD-PCR and conventional PCR. Among the samples with mutations detected by LiPA, COLD-PCR could detect mutations in more samples than conventional PCR. In particular, there were 35 samples with rtM204V/I mutations detected by COLD-PCR but not by conventional PCR, while all samples with mutations detected by conventional PCR were also detected by COLD-PCR. Thus, COLD-PCR was more sensitive than conventional PCR in detecting drug resistance mutations.

Although COLD-PCR may not be superior to the LiPA assay, it has several advantages. First, it is capable of enriching and detecting minority variants at all possible positions within the amplicon, whereas

LiPA is confined to a pre-defined set of variants. Second, compared with conventional PCR, it does not incur extra cost and is considerably cheaper than the LiPA assay. Third, compared with conventional PCR, COLD-PCR does not increase the run time of experiment.

Nevertheless, COLD-PCR has certain limitations. First, it may enrich minority species at other positions, thereby increasing the chance of obtaining high background sequencing noise. COLD-PCR may also induce a higher rate of PCR amplification error. This shortcoming can be overcome by using polymerases with proof-reading activities. In addition, as numerous possible quasispecies can be found in HBV clinical isolates, a single empirically determined T_c may not be applicable to all possible viral variations within the quasispecies population. Nevertheless, it is expected that this experimentally determined T_c would at least enhance the detection of minor mutations, and this was proven in the present study with both cloned plasmids and clinical specimen.

In a research setting, there are other more advanced methods to detect minor mutations, such as mass spectrometry, DNA microarray, next-generation sequencing, or ultradeep pyrosequencing. These methods are generally more expensive than COLD-PCR, especially when the number of samples tested is small. COLD-PCR is an affordable choice in the sensitive detection of minor mutations, especially in under-developed areas.

Conclusion

This study demonstrated that COLD-PCR could sensitively detect HBV drug resistance mutations at a level of 5-10% of the total viral population. It is simple and inexpensive and has the advantage of detecting novel mutations along the HBV reverse transcriptase gene.

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Results of this study have been published in: Wong DK, Tsoi O, Huang FY, et al. Application of coamplification at lower denaturation temperature-PCR sequencing for early detection of antiviral drug resistance mutations of hepatitis B virus. J Clin Microbiol 2014;52:3209-15.

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Differential gene expression profile of CD4+/CD8+ T cells in patients with hepatitis C virus and/or human immunodeficiency virus infection

L Yi, J Zhao, J Lu, Y Chen, D Huang, YF Li, L Chen, ZR Yang, HF Kung, ML He *

KEY MESSAGES

1. Coinfection of a host organism with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) alters the course of infection of each virus.
2. Treatment-naive HIV/HCV mono-/co-infected patients with CD4+ T cell count >300/μL were recruited, and the gene expression profile of their CD8+ and CD4+ T cells was investigated by microarray assay, bioinformatics analysis, and quantitative real time PCR validation.
3. Genes involved in cell proliferation, activation, differentiation, and regulation and cytokine responses were significantly altered in CD8+ T cells. In CD4+ T cells, innate immune response, cell cycle regulation, GPCR signalling pathway, transcriptional regulation, and metabolic pathways were significantly affected by HIV/HCV co-infection, compared with HCV mono-infection.
4. These findings offer new insight into disease progression in HIV/HCV co-infection, and may help to identify new markers for its management.

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Introduction

Co-infection with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) is common because of their similar routes of transmission (eg drug injection).[1] HIV co-infection significantly affects the natural history of hepatic fibrosis in HCV-infected persons.[2] Concomitant HIV infection increases the evolution of HCV quasispecies, the level of viraemia, and the extrahepatic viral reservoirs.[3] HIV viral load displays additive effects on HCV-triggered mitochondrial DNA depletion that may be associated with nucleoside analogue toxicity.[4] Although highly active antiretroviral treatment is effective for treating HIV mono-infection, its impact on HCV is controversial.[5] Co-infected patients have an increased risk of hepatotoxicity after such treatment.[6] Similarly, the toxicity of interferon-based HCV therapies is also exacerbated in HIV/HCV co-infected patients with a higher rate of relapse.[7] The underlying mechanisms of highly increased mortality remain elusive.

In this study, the gene expression profile of CD8+ and CD4+ T cells from treatment-naïve HIV/HCV mono- and co-infected individuals was analysed. Using gene set enrichment analysis, a network of enriched pathways related to the pathogenesis of disease progression in the co-infected patients was identified and confirmed by quantitative real-time PCR (qRT-PCR) assays. These findings offer new insight into the impact of co-infection at the gene transcription level.

Methods

This study was conducted from November 2009 to October 2011. A Chinese population with CD4+ count >300/μL was recruited from an ongoing voluntary-based HIV/AIDS surveillance study in Shenzhen, China from September 2009 to December 2010. The status of HCV, HIV, and HBV was strictly monitored according to the stringent guidelines of the Chinese Center for Disease Control and Prevention.

Fresh CD4+ and CD8+ T cells were isolated from 30 mL whole blood by microbead immunoselection according to the manufacturer's instructions (Miltenyi Biotec, Oslo, Norway). In each patient, equal amounts of CD4+ or CD8+ T cells were pooled together to form three biological replicas in each group. The remaining CD4+ or CD8+ T cells were separately stored at -80°C for validation. RNA was isolated using RNAeasy Total RNA Isolation Kit (Qiagen, Germany) and applied for microarray assays.

Transcriptomic analysis by microarray assays was performed using Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara [CA], USA). The images were acquired by the Affymetrix Scanner 3000 7G Plus and the CEL files were imported into the program Partek Genomic Suite

(version 6.4, Partek, St Louis [MO], USA) and the robust multi-chip average was normalised. Two-way ANOVA test was applied to identify differentially expressed genes (fold change >2 and adjusted P<0.05).

Gene set enrichment analysis (version 2.0, Broad Institute, Cambridge [MA], USA) was conducted. A nominal P value was calculated by permuting the genes 1000 times. Gene sets were collected from online databases such as Bio-Carta, React, and Kyoto Encyclopedia of Genes and Genomes (KEGG).

The qRT-PCR assay was carried out using ABI 7500 Real-Time PCR system with Power SYBR Green Master Mix (Applied Biosystems, Foster City [CA], USA).

Nonparametric test was used for pairwise comparisons. A P value of <0.05 was considered statistically significant.

Results

Differentially expressed genes in CD8+ T cells

A total of 28 869 genes were scanned. Of 110 transcript identifiers (IDs) differentially expressed in the HCV and HIV mono-infected groups, 81 were known genes (40 up-regulated, 41 down-regulated) and 29 were transcript IDs without known functions. Of these, 72 transcript IDs were differentially expressed in the HCV/HIV co-infected and the HCV mono-infected groups. The expression level of 47 of 55 genes in HCV/HIV co-infected group was lower than in the HCV mono-infected group. The Gene Ontology annotation assignment of these 55 differentially expressed genes was similar to those identified in HIV and HCV mono-infected groups. In the biological process category, biological regulation (64.15%), response to stimulus (49.06%), and metabolic process (47.17%) were the most dominant subcategories. In the molecular function and cellular component categories, cytokine binding, molecular transducer, and plasma membrane were significantly enriched. The KEGG pathways showed altered expression between the HCV/HIV co-infected and the HCV mono-infected groups including cytokine-cytokine receptor interaction, cell adhesion molecules, lysosome, chemokine signalling pathway, and antigen processing/presentation. In a Venn diagram for the gene expression relationship among the HIV/HCV mono-/co-infected groups (Fig), 18 IDs (17 genes, 1 transcript IDs) were shared between circles H and J and referred to genes that had ≥2-fold change in expression in both the HIV mono-infected and the HCV/HIV co-infected groups, compared with the HCV mono-infected group. In HIV mono-infection, 8 IDs (1 gene, 7 transcript IDs) were differentially expressed, compared with HCV mono-infection or HCV/HIV co-infection. Comparing the gene expression profile of the HIV/HCV co-infected group with the HIV or HCV mono-infected groups, 3 IDs (1 gene, 2 transcript IDs) displayed similar expression changes.

To confirm the observations from the microarray analysis, the mRNA level of the selected differentially expressed genes was measured using qRT-PCR. Blood samples from uninfected males with matched age were used as controls. Differentially expressed genes in the KEGG pathway of cytokine-cytokine receptor interaction were selected for qRT-PCR confirmation, as this pathway was enriched when comparing HIV with HCV mono-infection (R=9.06, 4 genes: CXCL16, TNFRSF9, CCR4 and CX3CR1) and HCV/HIV co-infection with HCV mono-infection (R=25, 8 genes: CXCL16, TNFRSF9, CCR4, CX3CR1, IL13RA1, IFNGR2, CD40LG, and PPBP). The mRNA isolated from CD8+ T cells was used for qRT-PCR experiments. The expression profile of 10 genes (CCR4, CD40LG, CX3CR1, CXCL16, IFNGR2, IL13RA1, EGR1, KLF4, GPR56, and TNFRSF9) exactly matched the observation from the microarray assays; another three genes (PPBP, OAS1, and P2RY13) matched at least one expression pattern out of three pairwise comparisons.

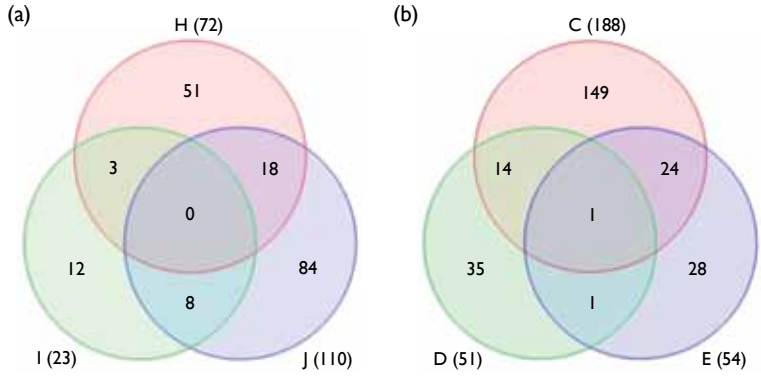

FIG. Venn diagram for differentially expressed transcript identifiers in (a) CD8+ T cells and (b) CD4+ T cells in paired comparisons of HCV-mono- versus HIV-mono-infection (circles J and E), HCV/HIV-co-infection versus HCV-mono-infection (circles H and C), and HCV/HIV-co-infection versus HIV-mono-infection (circles I and D)

Differentially expressed genes in CD4+ T cells

The number of differentially expressed transcript IDs in each comparison was identified. Of the 54 differentially expressed transcript IDs identified following comparison between HCV and HIV mono-infected groups, 24 showed significant matches with known genes involved in both immune system development and immune response (CD38, CXCL10, OAS3, FCGR3A, and IFI44L) and G-protein coupled receptor protein signalling pathway (P2RY13, GPR56, CX3CR1, and FFAR2). In HCV/HIV co-infection versus HCV mono-infection, 72 genes with known function were identified; 16 of them played important roles in stimulus response and the immune system process, including IL6,

FCGR1C, RSAD2, LILRA3, C1QC, IGSF6, IL1RN, and OAS3. In addition, genes regulating locomotor behaviour (such as CCRL2, FPR2, and IL8RA) also had differential expression profiles. Comparing HCV/HIV co-infection and HIV mono-infection, only seven genes were identified, including three encoding small nucleolar RNAs and four encoding AQP9, TNFAIP6, IL6, and PTX3. In a Venn diagram for the gene expression relationship among the HIV/HCV mono-/co-infected groups (Fig), only one cDNA clone was significantly differentially expressed among all three groups. The 24 IDs (8 cDNA clones and 16 genes, including IFIT1, FAM72D, SERPING1, FCGR3A, and SIAE) were found to have >2-fold change in expression in both the HIV mono-infected and the HCV/HIV co-infected groups, compared with the HCV mono-infected group. Comparing the gene expression profile of HIV/HCV co-infected patients with HIV or HCV mono-infected patients, 14 IDs (10 cDNA clones and 4 genes: SNORD116-6, SNORA38B, IL6, and PTX3) displayed similar expression changes.

Interestingly, the majority of gene sets up-regulated in HIV mono-infection also showed enrichment in HIV/HCV co-infection when compared with HCV mono-infection. Based on the biological functions, they could be mainly divided into cell cycle, immune response, and gene expression (regulation). In the cell cycle category, 32 of 88 and 18 of 67 gene sets were significantly up-regulated in the HIV mono-infected and HIV/HCV co-infected groups, respectively. The leading edge analysis revealed that the majority of them engaged in G1/S and G2/M transitions. Genes involved in the innate immune response, particularly in pathogen-associated molecular pattern recognition, showed increased expression in HIV mono-infection and contributed most to ES. In the HIV/HCV co-infected group, the most generally up-regulated gene sets were innate immune signalling including natural killer cell mediated cytotoxicity, toll-like receptor signalling pathways, NOD-like receptor signalling pathways, and complement activation.

In addition, the gene sets involved in signal transduction were important. Almost the entire gene set could be directly or indirectly associated with GPCR signalling. The mRNA levels from each paired comparison were randomly selected and validated by qRT-PCR.

Discussion

This study evaluated the global gene expression pattern of HCV/HIV co-infection against mono-infection in primary CD8+ and CD4+ T cells. Both HIV and HCV have evolved their own pathogenesis; cellular processes in the HIV/HCV mono-/co-infected patients were altered. This played a crucial role in cell proliferation/activation/differentiation; T cell regulation and cytokine responses were noted in CD8+ T cells. Gene sets involved in cell cycle progression, innate immune response, and some transcription factors in CD4+ T cells were affected mainly by HIV, whereas genes associated with extracellular matrix, beta cell development, and insulin synthesis and secretion were the major targets of HCV. Metabolic pathways appeared to be modulated by both viruses. In addition, the role of GPCR signalling pathway during HIV/HCV infection was revealed.

Future studies should validate the genes involved in T cell proliferation (such as TNFRSF9, CD160, CD38, CDCA7, CCNA2, and NUSAP1) and cytokine production that induces an innate immune response (such as MS4A1, CD40LG, and IL13RA1). As long as one gene is differentially expressed, the correlation between its expression level and disease status or progression should be investigated, as should its biological functions in cell culture or animal models using loss-of-function (such as RNA interference) and gain-of-function (such as lentiviral vector mediated gene transfer) studies. This will enable identification of biomarkers for better management of HCV/HIV co-infected patients and reveal new drug targets.

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Characterisation of novel anti-HIV/tuberculosis natural product analogues

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KEY MESSAGES

1. 10-chloromethyl-11-demethly-12-oxo-calanolide A (F18) is an effective antiretroviral compound *in vitro*.
2. F18 remains effective against HIV-1 strains that contain various mutations rendering them resistant to non-nucleoside reverse-transcriptase inhibitors.

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Introduction

Non-nucleoside reverse-transcriptase inhibitors (NNRTIs) are one of the key components of antiretroviral drug regimens against human immunodeficiency virus type 1 (HIV-1) replication. (+)-calanolide A, which is purified from the rainforest plant *Calophyllum*, is an anti-HIV-1/tuberculosis drug. In our previous study, a library of (+)-calanolide A was constructed and one novel compound 10-chloromethyl-11- demethly-12-oxo-calanolide A (F18) with improved antiviral activity was identified. This study investigated antiviral breadth, drug-resistance profile, and underlying mechanism of action of F18.

Methods and results

This study was conducted from March 2010 to February 2012.

In vitro selection of F18-resistant virus

HTLV-1 transformed human T-cell leukaemia (MT-2) cells were infected with 10^4 $TCID_{50}$ $HIV\text{-}1_{NL4\text{-}3}$ wild-type virus and cultured in 2-fold increasing concentrations of F18 ranging from 0.14 nM to 20 μM for 120 days. Virus replication was monitored based on syncytium formation. Ten resistant strains were selected (Table 1). Genotypic analysis showed that seven of ten variants contained a single mutation of amino acid Leu to Ile at position 100 in the RT gene. In addition, mutations of V292I, K103N, Y188H, V106I, T139R, and P225H also emerged in three other strains following F18 treatment.

To confirm their resistance and cross-resistance profile, these ten variants were assessed in MT-2 cells in the presence of F18, nevirapine (NVP), efavirenz (EFV), and etravirine (ETR). All mutant viruses were resistant to F18 (Table 1). The mutant viruses with mutations L100I or Y188H retained high sensitivity to the other three NNRTIs. K103N-containing virus resulted in more than a 350-fold change in EC_{50} to EFV and NVP, but was sensitive to ETR, whereas the HIV-1 variant with V106I/WT and T139R showed moderate resistance to EFV, NVP, and ETR. Therefore, F18-resistant HIV-1 mutations occurred in *in vitro* long-term culture of MT-2 cells infected with wild-type virus in the presence of F18, and these mutations conferred some cross-resistance to other NNRTIs.

Antiviral activity of F18 against NNRTI-resistant pseudovirus

To confirm the relevance of these *in vitro*-induced resistant mutations, a panel of seven NNRTI-resistant site-directed mutagenesis (SDM)-generated pseudoviruses for a GHOST (3)-CCR5 cell-based assay was established. Mutations that were induced by *in vitro* F18 selection were engineered into a clean $HIV\text{-}1_{NL4\text{-}3R\text{-}E\text{-}Luc}$+ pseudovirus backbone by SDM. Sensitivity of these mutant viruses was determined by infection with GHOST (3)-CCR5 cells in the presence of F18, NVP or (+)-calanolide A, and by measuring the degree of inhibition compared with infection with a wild-type virus (Fig 1a). Virus constructed with the minor Y188H mutation displayed a >100-fold higher level of resistance to F18 or (+)-calanolide A, compared with NVP, whereas virus containing the dominant

TABLE 1. Selection scheme and genotypic analysis of the reverse transcriptase of mutant HIV-1 strains that emerged under dose-escalating treatment of HIV-1_{NL4-3} wild-type virus with F18

Strain No. (No. of days in cell culture)	F18 concentration at which the selection initiated and the resistant strain selected	Mutation(s) in HIV-1 RT*	Fold change†			
			F18	NVP	EFV	ETR
1,4,6,7,8,9,10						
0	0.14 nM					
84	312 nM	L100I				
128	20 μM	**L100I**	>128	1.67	2.05	1.37
2						
0	0.14 nM					
91	625 nM	Y188H/WT				
105	2.5 μM	Y188H				
128	20 μM	Y188H	>128	3.75	0.004	0.34
3						
0	0.14 nM					
98	1.25 μM	T139R/WT				
120	10 μM	V106I/WT, T139R				
128	20 μM	V106I/WT, T139R	121	36.5	18.06	20.15
5						
0	0.28 nM					
91	1.25 μM	P225H				
98	2.5 μM	K103N/WT, P225H/WT				
105	5 μM	K103N				
112	10 μM	K103N, P225H/WT				
120	20 μM	K103N, P225H/WT, V292I	>128	>357	568.45	2.79

* The complete protease and reverse transcriptase genes were sequenced. The predominant mutation is underlined and in bold

† Calculated as the ratio between the EC_{50} of the compound for the mutant strain and the HIV-1_{NL4-3} wild-type strain obtained in the same experiment at the end of culture. EC_{50} of F18, NVP, EFV, and ETR against HIV-1_{NL4-3} wild-type virus is 41.0±2.0 nM, 42.0±1.7 nM, 0.1±0.003 nM, and 0.1±0.002 nM, respectively. Each result is the mean for a single experiment conducted in triplicate

mutation (7 of out 10 strains) L100I induced by F18 *in vitro* resulted in a 30- and 10-fold change in EC_{50} to F18 and (+)-calanolide A, respectively (Table 1). NVP susceptibility was noted with L100I mutant viruses. The novel mutation T139R found by F18 selection *in vitro* showed cross-resistance to all three compounds NVP, F18, and (+)-calanolide A. To a lesser extent, viruses with K101E and K103N mutations also displayed cross-resistance to F18, NVP, and (+)-calanolide A. Interestingly, virus with double mutations K103N and P225H exhibited high resistance to NVP with more than a 100-fold change in EC_{50}, but resistance to F18 and (+)-calanolide A was abolished when compared with the single mutation K103N.

As cross-resistant mutations may exist among various NNRTIs, susceptibility of ten additional HIV-1 NNRTI-resistant SDMs to F18, NVP, and (+)-calanolide A, alone or in combination, was determined (Fig 1b). These mutations were previously reported based on the current NNRTIs in clinical use. One of the most prevalent mutations among antiretrovial-therapy-experienced patients was Y181C, and virus with this mutation was sensitive to F18 with an EC_{50} of 1.0 nM and a reduced susceptibility to NVP for more than a 100-fold difference in EC_{50}. Double mutant with K103N/Y181C, Y188L and V106A/G190A/F227L mutants were cross-resistant to the antiviral effects of the three NNRTIs.

Antiviral activity of F18 in combination with some US-FDA-approved antiretrovirals

Given the unique antiviral features of F18, its efficacy when used in combination with each of eight US-FDA-approved drugs using PBMC assays was determined. There were no antagonistic effects for any combinations of F18 plus another drug against HIV-1_{NL4-3} infected PBMCs (Table 2). Most combinations showed a slightly or highly synergistic effect as calculated by the Prichard and Shipman MacSynergy II software.

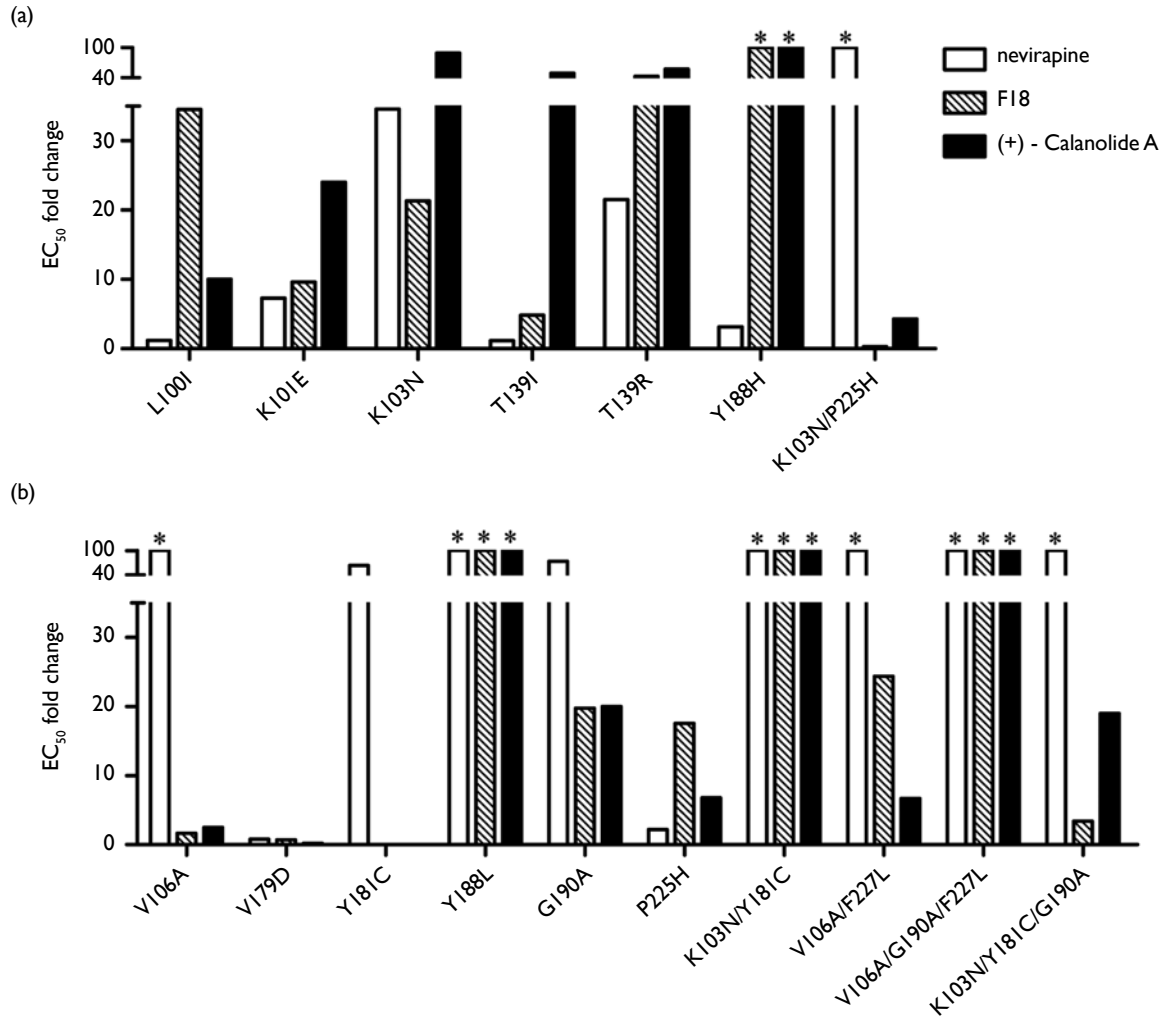

FIG. Resistance of constructed HIV_{NL4-3} SDMs to different non-nucleoside reverse-transcriptase inhibitors (NNRTIs): GHOST (3)-CCR5 cells were infected in the presence of NVP, F18, or (+)-calanolide A with NNRTI mutant pseudoviruses constructed by SDM for (a) mutations identified from Table 1, (b) mutations with resistance to different classes of antiretroviral drugs. The average EC_{50} fold changes are shown for triplicate experiments.
* EC_{50} is not reached at the concentration tested

Discussion

One of the major obstacles to the development of NNRTIs is the extensive cross-resistance within this class of antiretrovirals. F18 displayed a unique profile of cross-resistance. In contrast to NVP, F18 remained effective against HIV-1 SDMs containing NNTRI-resistant mutations T139I, Y181C, V179D, V106A, K103N/P225H, and K103N/Y181C/G190A. From our F18-resistance induction study, F18 did not readily induce NVP-resistant or (+)-calanolide A-resistant viruses, but the resultant dominant L100I mutation attributed to F18-resistance, which is similar to another (+)-calanolide A stereoisomer–dihydrocostatolide.[1] This finding suggests that F18 may function more similarly to dihydrocostatolide than (+)-calanolide A. In addition, due to structural similarity, F18 and (+)-calanolide A shared a similar profile against most NNRTI-cross resistant viruses (Fig). These two drugs also displayed their unique characteristics. For example, F18 was a potent inhibitor against (+)-calanolide A-induced T139I mutant. Conversely, (+)-calanolide A inhibited F18-induced L100I mutant more effectively. These results suggest that the major binding moiety of F18 engages HIV-1 RT at a different binding motif towards L100 when compared with T139 usage of (+)-calanolide A. In addition, most of the resistant viruses including L100I selected by F18 can be inhibited by NVP, EFV, and ETR, indicating a low level of cross-resistance between F18 and these three

TABLE 2. Antiviral effect of F18 in combination with eight US-FDA-approved antiretroviral compounds against HIV-1_{NL4-3} in PBMC assays

Drug	Mean synergy volume*	Antiviral effect
Nucleoside reverse transcriptase inhibitors		
Zidovudine	87.62	Slightly synergistic
Lamivudine	17.32	Additive
Stavudine	27.71	Additive
Didanosine	297.47	Highly synergistic
Non-nucleoside reverse transcriptase inhibitors		
Nevirapine	73.1	Slightly synergistic
Efavirenz	4.01	Additive
Protease inhibitors		
Nelfinavir	116.85	Highly synergistic
Integrase inhibitors		
Raltegravir	54.12	Slightly synergistic

* Positive values represent the synergistic interaction between F18 and other antiretroviral drugs

FDA-approved NNRTIs (Table 1). We conclude that the drug-resistance profile of F18 is distinctly different from that of other NNRTIs,[2-4] possibly due to a distinct binding motif of F18 to HIV-1 RT that differs to other NNRTIs.

Since the discovery of NNRTIs, combination therapy of at least three antiretrovirals has become the gold standard for clinical treatment of HIV-1 patients in the last 20 years. To avoid issues of drug-drug interaction, we tested the combined anti-HIV activity of F18 with each of eight commonly used antiretrovirals. As a natural product-derived small molecule, F18 had no antagonistic effect when used in two-drug combination against both wild-type and drug-resistant viruses (Table 2). Interestingly, the lack of antagonistic effects between F18 and NVP or EFV provided further evidence that F18 does not share identical binding motifs to HIV-1 RT with either NNRTI.

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Sophora flavescens (Ku-Shen) as a booster for antiretroviral therapy through cytochrome P450 3A4 inhibition

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KEY MESSAGES

1. *Sophora flavescens* extract dose-dependently inhibited human hepatic CYP3A4 activity. The ethyl acetate (EA) fraction containing prenylated flavonoids was most effective.
2. *Sophora* EA fraction slightly inhibited the efflux of indinavir from the basolateral to apical side in a Caco-2 cell monolayer model.
3. Unlike ritonavir, coadministration of *Sophora* EA fraction did not enhance plasma indinavir concentration.
4. Treatment with *Sophora* total extract significantly decreased plasma exposure of indinavir, associated with intestinal and hepatic P-gp induction, and upregulation of CYP3A activity.
5. Patients prescribed indinavir should be cautioned about intake of *S flavescens* extract or *Sophora*-derived products.

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Introduction

Combination pharmacotherapy for the treatment of human immunodeficiency virus (HIV) is effective for viral load reduction and clinical success.[1] Nonetheless, HIV drugs are limited by their low bioavailability, limited central nervous system penetration, and undesirable side effects. One factor resulting in low bioavailability is the drug's susceptibility to metabolism by cytochrome P450 (CYP), in particular CYP3A4, a predominant CYP subfamily in human liver. Thus, concurrent use of CYP inhibitors (eg ritonavir) and a protease inhibitor (eg lopinavir) is the preferred highly active antiretroviral regimen.[1] The enzyme inhibitor acts as a 'bioavailability booster' by inhibiting CYP3A4 that metabolises the protease inhibitor drug molecules. We hypothesised that Chinese herbs that possess CYP3A4 inhibitory activity may potentiate (boost) the therapeutic effects of anti-HIV drugs. In our preliminary study, *Sophora flavescens* was the most effective in CYP3A4 inhibition among 50 herbs tested.[2] To establish biological evidence to support the use of *Sophora flavescens* as an adjuvant (booster) in the treatment of HIV, an *in vitro* CYP3A4 inhibition assay was established. Using a probe substrate method, the CYP3A4-inhibitory effects of *Sophora* extract and its ingredients were evaluated. This study also determined the effect of *Sophora* on P-gp in a Caco-2 cell monolayer model. In addition, an animal system was used to measure the *in vivo* effect of *S flavescens* on the oral pharmacokinetics of indinavir, a typical protease inhibitor in anti-HIV therapy. The possible involvement of P-gp and CYP3A in this interaction was examined by measuring their intestinal and hepatic mRNA/protein level and enzyme activity.

Methods

This study was conducted from November 2009 to October 2011. Dried root of *Sophora flavescens* was extracted by heating with 70% ethanol. The extract was filtered, concentrated by rotary evaporation, and freeze-dried to obtain a powder that contained approximately 11.1% oxymatrine. Sequential liquid extraction with ethyl acetate (EA) and butanol was performed to produce EA-, butanol- and water-soluble fractions. *Sophora* EA fraction was further separated using an open-pressure column embedded with Sephadex packing and preparative HPLC to produce nine compounds. The purity of isolated compounds was higher than 97% as determined by HPLC, and their identity was established by MS and NMR analysis.

CYP3A4 activity was determined by 6β-hydroxylation of testosterone. Briefly, a reaction mixture containing testosterone, potassium phosphate buffer, pooled human liver microsomes, an NADPH-generating system, and various concentrations of crude extracts, fractions or pure compounds was incubated at 37°C for 30

minutes. After sample extraction with EA, 6β-hydroxytestosterone was analysed using a HPLC method.

The effect of *Sophora* EA fraction on intestinal dual transport of indinavir was determined in Caco-2 cell monolayers grown in Transwell inserts for 21 days. Transfer buffer containing indinavir or indinavir plus *Sophora* EA fraction was added to the apical or basolateral chamber. Aliquots were taken from the basolateral or apical chamber at 20, 40, 60, 90, 120, 180 minutes.

Rats were orally gavaged with indinavir (40 mg/kg), and an hour later with 1.5% Tween 80 (vehicle), ritonavir (10 mg/kg), Marine capsule (45 mg/kg of oxymatrine equivalent), *Sophora* extract (0.158 or 0.63 g/kg), or *Sophora* EA fraction (82 mg/kg, 0.164 g/kg or 0.328 g/kg), respectively. All treatments were given twice a day for 7 days to reach a steady state and mimic the combined use of a protease inhibitor and ritonavir in humans. On day 8, blood samples were collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, and 5 hours after dosing by orbital bleeding under anaesthetic with isoflurane. Plasma was obtained by centrifugation. After alkalisation and extraction with EA, indinavir was separated by a C_{18} column and determined by APCI-MS using midazolam as an internal standard (Fig).[3]

The amounts of mRNA that encoded CYP3A1, CYP3A2, and P-gp (mdr1a and mdr1b) in the intestine and liver were quantified by real-time PCR. Total RNA was extracted using chloroform and isopropanol, and then converted to cDNA using a high-capacity cDNA reverse transcription kit. Taqman assays using specific primers were performed for the quantification of mRNA in an ABI step-one real time PCR system. The relative mRNA levels were calculated by the $2^{-\Delta\Delta CT}$ method.[3]

Intestinal brush border membrane, intestinal mucosa homogenate and liver microsomes were used for protein analysis. Protein content of CYP3A and P-gp was quantified by western-blotting using specific antibodies.[3]

Hepatic CYP3A activity was measured by a luminescent assay (P450-Glo) according to the manufacturer's instructions (Promega, WI, USA).[3]

Results

Sophora flavescens extract caused dose-dependent inhibition of human hepatic CYP3A4 activity. The EA fraction was most effective in inhibiting CYP3A4, followed by butanol fraction and water fraction. Nine CYP3A4-inhibitory flavonoids were further isolated from the EA fraction (Table 1). Sophoraflavanone G was the most potent inhibitor, with an IC_{50} of 4.83 μg/mL. *Sophora* alkaloids did not show any significant effect. *Sophora* EA fraction also slightly inhibited the efflux of indinavir from the basolateral to apical side in the Caco-2 cell

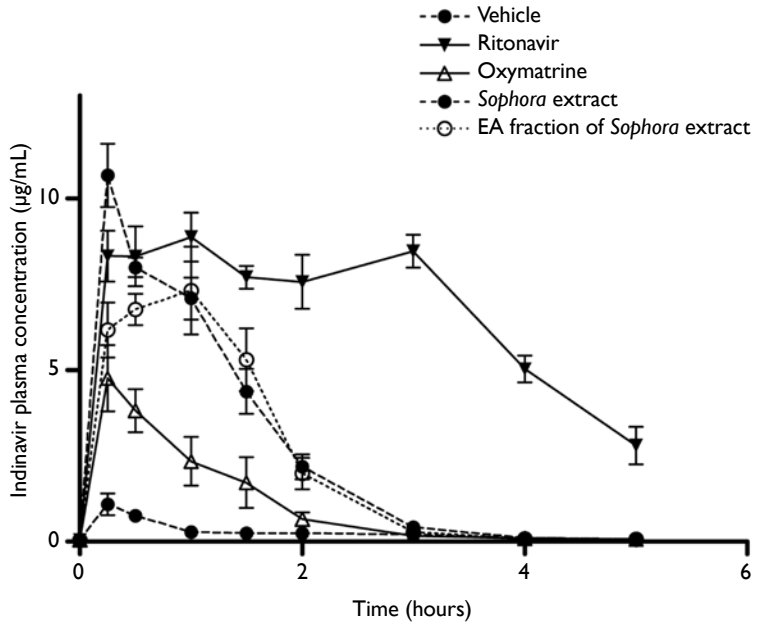

FIG. Plasma concentrations of indinavir: animals received oral administration of indinavir (40 mg/kg) together with 2% Tween 80 (vehicle), ritonavir (10 mg/kg), oxymatrine (45 mg/kg), 70% ethanol extract of *Sophora flavescens* (0.63 g/kg), or ethyl acetate (EA) fraction of the *Sophora* extract (82 mg/kg) twice a day for 7 days, respectively. Values are expressed as mean±SEM (n=6)

TABLE 1. Inhibitory effects of *Sophora* extract and fractions, the major alkaloid components, and the isolated flavonoids on CYP3A4 activity in pooled human liver microsomes

Parameter	Mean
Inhibitory effects (IC_{50}) [μg/mL]	
Total extract	51.2
Water fraction	>3000
Butanol fraction	47.2
Ethyl acetate fraction	5.2
Major alkaloid components (μM)	
Matrine	>500
Oxymatrine	>500
Sophoridine	>500
Sophocarpine	>500
Ketoconazole	0.126
Isolated flavonoids on CYP3A4 activity in pooled human liver microsomes (IC_{50}) [μg/mL]	
Sophoraflavanone G	4.8
Kushenol I/N	25.4
Kuraridin	10.5
2'-Methoxykurarinone	19.3
Leachianone A	5.5
Kushenol A	33.9
Kushenol X	8.43
Kushenol L	7.33
Xanthohumol	13.1

TABLE 2. *In vitro* and *in vivo* effects of *Sophora flavescens* and ritonavir (a positive booster) on CYP3A4 and P-gp, and the impact on plasma exposure of indinavir

Effect	*Sophora* extract	Ethyl acetate fraction	Oxymatrine	Ritonavir
In vitro CYP3A4 activity	Inhibition (IC_{50}=51.2 μg/mL)	Inhibition (IC_{50}=5.2 μg/mL)	No effect	Inhibition (previously reported)
P-gp mediated indinavir efflux	-	Slight inhibition	-	Inhibition (previously reported)
Plasma indinavir concentration	Decrease (up to 83% in AUC)	No effect	Decrease (up to 61% in AUC)	Increase (2.5-fold in AUC)
In vivo CYP3A activity	Upregulation	No effect	No effect	Inhibition
CYP3A expression	Induction	-	-	-
P-gp expression	Induction	-	-	-

monolayer model, indicating that it might increase intestinal absorption of indinavir.

In a rat model, co-administration (7 days) with ritonavir (a potent CYP3A4 inhibitor) significantly increased plasma exposure of indinavir, which was associated with inhibition of hepatic CYP3A activity (Table 2). Nonetheless, unlike ritonavir, *Sophora* EA fraction did not enhance plasma indinavir concentration. Co-administration with *Sophora* total extract markedly decreased plasma exposure of indinavir with a reduction in AUC and C_{max}, values. Oxymatrine was likely one of components responsible for this herb-drug interaction. The decreased indinavir exposure by *S flavescens* was, at least partly, attributed to the intestinal and hepatic P-gp induction, and upregulation of CYP3A activity.

Discussion

In the treatment of HIV infection, it is common practice to boost the protease inhibitor effect using a CYP3A4 inhibitor. The combined use of herbs and prescribed drugs can potentially attenuate drug efficacy and/or enhance toxicity. Prolonged co-administration of health products, such as St John's wort, leads to a decrease in plasma indinavir concentration.[4]

To determine the potential of *Sophora flavescens* as a booster for antiretroviral therapy, the present study established an experimental system that combined an *in vitro* CYP3A4 inhibition assay and *in vivo* pharmacokinetic study. *In vitro* findings implied the boosting potential of *Sophora* EA fraction, but *in vivo*, indinavir showed no enhancing effect on plasma levels in rats, and CYP3A activity was also not affected by treatment with an EA fraction.

Sophora EA fraction contains a mixture of prenylated flavonoids. Sophoraflavanone G was the most effective isolated compound in inhibiting CYP3A4, but it was still much less potent than ritonavir. Thus, the *in vivo* study used a high dose of EA fraction (maximum: 0.656 g/kg/day), which was comparable with ritonavir in terms of *in vitro* CYP3A4 inhibition. As such, the inability of EA fraction to boost indinavir is not a matter of dosage. The concentrations of *Sophora* flavonoids were quite low after oral administration of EA fraction. The discrepancy between *in vitro* and *in vivo* study in terms of EA fraction could be explained by the low oral bioavailability of the flavonoid components.

Co-administration of *Sophora* extract and indinavir (7 days) significantly decreased the plasma concentrations of indinavir. Moreover, co-administration with Marine capsule also decreased the plasma indinavir concentrations, with 61% reduction in $AUC_{0-\infty}$. Although there may be other ingredients present in the extract, oxymatrine is definitely a component in *S flavascens* and associated with the pharmacokinetic interaction with indinavir.

The bioavailability of indinavir is largely limited by efflux through intestinal P-gp and first-pass metabolism by CYP3A.[5] *Sophora* treatment could induce mRNA expression of CYP3A1 in the small intestine and liver. As *Sophora* alkaloids could induce CYP3A4 mRNA expression via activation of the pregane X receptor, the elevated CYP3A1 mRNA levels might also be caused by activation of this CYP3A-transcriptional regulator. Although CYP3A protein expression did not change, hepatic CYP3A activity increased after *Sophora* treatment. The up-regulation of CYP3A activity enhanced indinavir metabolism and hence facilitated drug elimination, which can, at least partially, explain the decreased plasma exposure of indinavir. Meanwhile, *Sophora* treatment dose-dependently increased mRNA level and protein expression of P-gp in the intestine and liver. Since intestinal and hepatic CYP3A and P-gp can be concurrently induced through the activation of common transcription factors—pregane X receptor and the constitutive androstane receptor, it is speculated that *Sophora* alkaloids may also induce P-gp. By extruding the substrate (indinavir)

during absorption or interplaying with CYP3A to further enhance intestinal first-pass metabolism, the induction of intestinal P-gp by *S flavascens* could lead to decreased plasma indinavir. It is evident that P-gp and CYP3A cannot fully account for the entire process of indinavir absorption and metabolism, and there must be other mediators that remain to be identified.

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Use of interferon gamma release assay to assess latent tuberculosis infection among healthcare workers in Hong Kong

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KEY MESSAGES

1. Overall baseline interferon gamma release assay positivity was 20.7%.
2. The conversion to interferon gamma release assay positivity at 3 months was 8.85% in the exposed group and 4.54% in the non-exposed group using the conventional cut-off of 0.35 IU/mL.
3. When grey zone results (0.2I-0.7 IU/mL) were included, the proportion of non-specific conversions and reversions could be reduced.
4. Interferon gamma release assay can be an adjunct tool in contact investigation of latent tuberculosis infection in healthcare workers.

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Introduction

Tuberculosis (TB) is a highly infectious airborne disease. Healthcare workers are at increased risk of contracting infection because of exposure to a concentration of infectious patients in their work environment. Contact investigations are hampered by the lack of specific markers. The tuberculin skin test (TST) has been used to diagnose latent tuberculosis infection (LTBI) with variable success, as the majority of local residents have been inoculated with Bacillus Calmette-Guérin (BCG) vaccine at birth.[1]

In-vitro interferon gamma release assay (IGRA) including QuantiFERON-TB Gold In-Tube (QFT-GIT) can identify individuals infected with TB who have been vaccinated with BCG. It is non-invasive, with no 'booster' effect from repeated testing, and less painful compared with TST. Nonetheless, its applicability to daily use including contact investigation remains unclear, owing to the complex biological basis of IGRA.

Methods

This was a prospective cohort study. Healthcare workers from three acute hospitals with or without unprotected exposure to smear-positive TB patients were recruited between 1 January 2010 and 30 June 2011 and followed up until 30 June 2012 using QFT-GIT, as per the manufacturer's recommendations.[2] The two groups were matched for sex, age, rank, and department. Outcome measures included the baseline positive rate, the conversion and reversion over time, and the associated factors.

The number of years as a healthcare worker and the exposure incident including nature and duration were recorded. Blood samples were taken at baseline, 3 months, 6 months, and 12 months. Any participants with ambiguous QFT-GIT results or symptoms suggestive of active TB were encouraged to seek medical advice from microbiologists of the investigation team. They were then referred to respiratory physicians for further assessment using sputum AFB smear, culture, and chest radiography.

Results

A total of 159 exposed and 120 non-exposed healthcare workers aged 22 to 63 (mean, 39.2) years were recruited. The mean number of years as a healthcare worker was 11.48 (range, 0.1-43) years. The female-to-male ratio was 82.4:17.6; 59.5% of participants were nurses. Participants were recruited from non-admission medical wards (26.2%), medical admission wards (23.3%), orthopaedic wards (24.4%), surgical wards (13.3%), and others (12.9%). Baseline characteristics of the two groups were comparable. A total of 46 TB contact investigations were performed.

The baseline QFT-GIT positivity for the exposed and non-exposed groups was 19.5% and 20.8%, respectively (relative risk [RR]=0.96, 95% CI=0.74-1.25, P>0.05). It was associated with age ≥40 years (RR=1.62, 95% CI=1.23-2.11), and working

as a healthcare worker for ≥10 years (RR=1.44, 95% CI=1.15-1.79). Department or staff type was not associated with baseline positivity.

Regarding conversion and reversion over time, the AFB smear positivity of the index patients was as follows: AFB + 34.5%, ++ 12.8%, and +++ 52.7%. The mean number of hours of exposure was 27.08 (range, 8-220). Using a conventional cut-off of 0.35 IU/mL, 142 and 113 subjects from the exposed and non-exposed group had >1 specimen for assessment of conversion or reversion, respectively. For the respective groups, conversion at 3 months was 8.85% (10/113) and 4.54% (4/88) (RR=1.30, 95% CI=0.91-1.85), whereas conversion was detected in 14.2% (16/113) and 13.6% (12/88), and reversion occurred in 25% (7/28) and 29.2% (7/24). When a grey zone of 0.2 to 0.7 IU/mL was implemented, 133 and 106 participants in the respective groups were eligible for analysis: conversion at 3 months was 2.97% (3/101) and 1.03% (1/79), whereas conversion during the study period was 4.95% (5/101) and 3.79% (3/79), and reversion occurred in 13.6% (3/22) and 0% (0/13).

Eight participants asked about their QFT-GIT results; two of them had sputum and chest radiographs examined and all were negative. No participant required consultation with a chest physician. One participant from the non-exposed group developed active TB. The three blood results were all >1 IU/ml. No participant from the exposed group had active TB.

Discussion

Conversion to positivity at 3 months was adequate to determine the infection status of contacts. The 3-month period is also the time frame required to exclude a false negative TST result following TB exposure.[3]

The overall baseline positivity rate was 20.7% using the conventional cut-off point. The exact significance of QFT-GIT positivity is not clearly understood. Although it is mostly implicated as diagnostic for LTBI, it can also represent a state of active TB infection, or even treated TB infection. QFT-GIT has consistently shown a higher specificity than TST, especially in BCG-vaccinated populations.

Conversion is generally interpreted as the acquisition of TB infection after a definitive history of TB exposure. False conversion can be due to

TABLE 1. Participant characteristics*

Characteristics	Overall (n=279)	Exposed (n=159)	Non-exposed (n=120)	P value (exposed vs non-exposed)
Age (years)	39.20±10.68 (63-22)	39.79±10.64 (58-22)	38.41±10.73 (63-22)	>0.05
No. of years as a healthcare worker	11.48±9.12 (43-0.1)	11.43±9.10 (37-0.1)	11.56±9.19 (43-0.5)	>0.05
Age-group (years)				>0.05
<30	62 (22.7)	34 (21.8)	28 (23.9)	
30-39	93 (34.1)	48 (30.8)	45 (38.5)	
40-49	55 (20.1)	35 (22.4)	20 (17.1)	
≥50	63 (23.1)	39 (25.0)	24 (20.5)	
No. of years as a healthcare worker				>0.05
<10	122 (46.2)	70 (47.3)	52 (44.8)	
≥10	142 (53.8)	78 (52.7)	64 (55.2)	
Sex				>0.05
Female	230 (82.4)	134 (84.3)	96 (80.0)	
Male	49 (17.6)	25 (15.7)	24 (20.0)	
Ward/department				<0.05
Medical	73 (26.2)	42 (26.4)	31 (25.8)	
Medical admission	65 (23.3)	44 (27.7)	21 (17.5)	
Orthopaedics	68 (24.4)	15 (9.4)	53 (44.2)	
Surgical	37 (13.3)	33 (20.8)	4 (3.3)	
Others	36 (12.9)	25 (15.7)	11 (9.2)	
Staff type				>0.05
Nursing	166 (59.5)	95 (59.7)	71 (59.2)	
Allied health, supporting, and others	113 (40.5)	64 (40.3)	49 (40.8)	

* Data are presented as mean±SD (range) or No. (%) of participants

TABLE 2. QuantiFERON-TB Gold In-Tube (QFT-GIT) test results

QFT-GIT test	No. (%) of participants			Relative risk (95% CI)
	Overall (n=279)	Exposed (n=159)	Non-exposed (n=120)	
Baseline				0.96 (0.74-1.25)
Positive	56 (20.1)	31 (19.5)	25 (20.8)	
Negative or indeterminate*	223 (79.9)	128 (80.5)	95 (79.2)	
Conversion to positivity in the 2nd blood sample				1.30 (0.91-1.85)
Yes	14 (6.9)	10 (8.8)	4 (4.5)	
No	189 (93.1)	104 (91.2)	85 (95.5)	

* Three indeterminate results (one from exposed and two from non-exposed group)

TABLE 3. Association between participant characteristics, exposure details, and baseline positivity

Variables	No. (%) of participants		P value (positive vs negative/ indeterminate)	Relative risk (95% CI)
	Baseline positive (n=56)	Baseline negative/ indeterminate (n=223)		
Exposure			>0.05	0.96 (0.74-1.25)
Non-exposed (reference)	25 (20.8)	95 (79.2)		
Exposed	31 (19.5)	128 (80.5)		
AFB result of the index patient			>0.05	-
Non-exposed	25 (20.8)	95 (79.2)		
+	7 (13.7)	44 (86.3)		
++	4 (21.1)	15 (78.9)		
+++	17 (21.8)	61 (78.2)		
Age (years)			<0.05	1.62 (1.23-2.11)
<40 (reference)	20 (12.9)	135 (87.1)		
≥40	33 (28.0)	85 (72.0)		
No. of years as healthcare worker			<0.05	1.44 (1.15-1.79)
<10 (reference)	15 (12.3)	107 (87.7)		
≥10	37 (26.1)	105 (73.9)		
Sex			>0.05	0.78 (0.39-1.56)
Female (reference)	48 (20.9)	182 (79.1)		
Male	8 (16.3)	41 (83.7)		
Ward/department			>0.05	-
Medical	14 (19.2)	59 (80.8)		
Medical admission	13 (20.0)	52 (80.0)		
Orthopaedics	13 (19.1)	55 (80.9)		
Surgical	6 (16.2)	31 (83.8)		
Others	10 (27.8)	26 (72.2)		
Staff type			>0.05	1.07 (0.76-1.51)
Nursing (reference)	32 (19.3)	134 (80.7)		
Allied Health, supporting and others	24 (21.2)	89 (78.8)		

concomitant illness, non-specific boosting, and fluctuation of IFN-gamma responses, as well as laboratory factors. When analysing risk factors for conversion at 3 months for the exposed group, age ≥40 was a significant risk (RR=1.96, 95% CI=1.30-2.96), raising the possibility of an immune-boosting phenomenon. No relationship between conversion and TB disease was identified; thus longer follow-

up is required. Reversion may be due to a 'false positive' in the first specimen, or spontaneous immune clearing of the infection with TB treatment. 'Wobbling phenomenon' may be due to one or more factors mentioned above. In one longitudinal study, contacts with precisely defined exposure in point-source outbreaks were monitored serially. Subjects who had transiently positive IGRA results were identified; there was a possibility that some contacts may have acquired, and spontaneously cleared, a transient *M tuberculosis* infection.[4] Nonetheless, an immune-boosting phenomenon is a more likely explanation. In the present study, transient change of positivity/negativity was noted in 16 participants.

Non-specific conversion and reversion is a cause of concern, as this might precipitate unnecessary LTBI treatment. Participants with results close to the cut-off value of 0.35 IU/mL tended to have more non-specific conversions and reversions. A high positive result of >1.0 IU/mL tended to remain positive. For this reason, a grey zone of 0.2-0.7 IU/mL is proposed. Results outside this zone are presumed to be true negatives or positives. In the present study, the use of a grey zone significantly reduced the number of conversions and reversions. Whether this reduction represents a true improvement in detection of true conversion and reversion has yet to be confirmed. In addition, the current FDA-approved package insert and management guidelines do not provide advice on interpretation of results in the grey zone.[5]

As the high positive result of >1.0 IU/mL tends to remain positive, quantitative measurements for IGRAs should be reported.[3] This may be highly relevant as the only participant that developed active TB disease had high values (>1 IU/mL). Nonetheless, there are no interpretive guidelines for the quantitative levels. The following approach to interpretation has been recommended: (1) quantitative results should not be used for prognostic or therapeutic monitoring purposes at this time, and (2) if the quantitative results are close to cut-off values, reversion or conversion is more likely upon re-testing.[5]

The most appropriate contact investigation procedure in Hong Kong hospitals remains controversial. In our study, the baseline LTBI rate was quantified as 20.7%. For IGRA, the exposed group had a tendency to convert at 3 months, regardless of the cut-off value, indicating an association between TB exposure and conversion. Despite the large cohort, none of the exposure events reached the threshold of AFB +++ with exposure ≥120 hours arbitrarily set by the infection control team of the Queen Elizabeth Hospital. Repeated immune boosting might be a significant cause of QFT-GIT positivity, as evidenced by the association of baseline positivity with age ≥40 years and working as a healthcare worker for ≥10 years, as well as more conversion with age ≥40 years. No clear association between QFT-GIT positivity and active TB disease was found, possibly owing to the short follow-up duration of one year.

Further studies to include longer term serial monitoring, other TB exposure events, and the prognostic implication of IGRA in the development of active TB disease are warranted.

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Expression analysis of putative small regulatory RNAs in *Mycobacterium tuberculosis:* effects of growth phase and oxidative stress

MTK Au *, EWC Chan, RCY Chan

KEY MESSAGE

Results from northern blotting and RT-PCR can reveal which putative genes are authentic small RNA-coding elements and provide preliminary data on the induction characteristics of these important genetic regulators, thereby extending the number of small RNAs in MTB that are experimentally detectable at a transcriptional level, and fostering future research to delineate their regulatory role in this pathogen.

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Introduction

Mycobacterium tuberculosis (MTB) is responsible for about 2.9 million deaths per year, and one third of the world's population are infected with MTB.[1] As an intracellular pathogen, MTB occupies different environmental niches and endures different growth conditions during the infection process. The bacterium responds to changing growth conditions by eliciting a series of adaptive responses that often entail spatial and temporal coordinated regulation of gene expression events that enable MTB to colonise the host, escape immune defence, scavenge for nutrients, and finally survive and proliferate in macrophages.[2] Thus, the pathogenicity of MTB is closely related to its ability to adjust gene expression patterns in the ever-changing environment in the host. Emerging evidence has revealed that small RNA molecules are the key gene expression regulators of adaptive stress responses in a wide range of bacterial pathogens.[3] These genetic elements exert their regulatory function by acting directly on target mRNAs by an antisense mechanism or act indirectly by interacting with regulatory proteins, thereby mediating the translation and/or the stability of mRNA transcripts that encode proteins with virulent functions, or proteins with important roles in adaptive responses during the infection process. In silico analysis of the genome sequence of MTB CDC 1551 has suggested a complex array of 47 putative small RNA-coding genes in this organism, highlighting the importance of these genetic elements in the bacterial adaptive responses during MTB infection.[4] Nevertheless, experimental evidence of the existence of small RNAs in this pathogen is scarce. This study aimed to analyse the possible occurrence of small RNAs in MTB. The presence and relative abundance of RNA transcripts of each of the putative small RNA-coding genes in MTB CDC 1551 was examined using northern blotting and RT-PCR. MTB cells were collected from cultures at different growth phases or after being subjected to hydrogen peroxide treatment for detection of small RNAs. Results from these experiments can reveal which putative genes are authentic small RNA-coding elements and provide preliminary data on the induction characteristics of these important genetic regulators, thereby extending the number of small RNAs in MTB that are experimentally detectable at a transcriptional level, and fostering future research to delineate their regulatory role in this pathogen.

Methods

This study was conducted from February 2010 to July 2011. The MTB clinical reference strain CDC 1551 was grown in Middlebrook 7H9 broth at 37°C. Exponentially grown CDC 1551 cultures were harvested at OD600 between 0.4 and 0.6; stationary cultures were harvested 1 week after OD600 had reached 0.8.

A total of 47 putative MTB CDC 1551 small RNAs were identified by the sRNAPredict2 program.[4] These putative small RNAs are located in the chromosomal intergenic regions of the MTB CDC 1551 genome, and their length ranged from 60 to 500 nucleotides.

MTB cells at different growth stages (exponential/stationary) or after being subjected to H_2O_2 challenge were harvested by centrifugation. Total RNA was purified from the cell pellets with Trizol (Invitrogen, CA, USA). To enrich the small RNAs in the preparation, the 16S and 23S rRNA species were removed from the total RNA using the MICROBExpress kit (Ambion, USA). For northern blot analysis, RNA samples were fractionated on denaturing gel. The relative abundance of 5S rRNA was used to standardise the loading amounts

between samples. After electrophoresis, RNA was transferred to Hybond-N+ membranes (Amersham Life Science, UK) by electroblotting. Expression of small RNAs was detected by hybridising the membranes with specific probes that were PCR amplified from the MTB CDC 1551 genomic DNA using oligonucleotide primer pairs covering entirely each of the putative small RNA-coding genes. The labelling of the probes with digoxigenin and the chemiluminescence detection of the hybridised small RNAs were performed using a commercially available kit (Roche Diagnostics, Switzerland). As a control, the expression of a constitutively expressed MTB gene, sigA,[5] was also detected in parallel.

Total RNA prepared by the Trizol method was treated with RNase-free DNase to remove potential DNA contamination. The purified RNA was reverse-transcribed using random hexamers in the SuperScript III Reverse Transcription kits (Invitrogen). PCR was performed using reagents from Qiagen (Germany). Reactions for the MTB housekeeping gene, sigA, were also run in parallel as control.

Cells of MTB CDC 1551 at the exponential phase were first harvested by centrifugation and re-suspended in a minimal volume of Middlebrook 7H9 broth. Ten times the volume of Middlebrook 7H9 broth with 20 mM H_2O_2 was then added to the cell suspension and incubated at 37°C for 16 hours. The isolation of total RNA and the detection of small RNA expression were conducted as described above.

Results

Detection of the expression of putative small RNAs in MTB CDC 1551 at different growth phases

Total RNA purified from exponential and stationary phase cultures of CDC 1551 was used for the detection of the 47 putative small RNAs. Using northern blotting and RT-PCR, the small RNA transcripts accumulated in these two growth phases were examined. Signals corresponding to the transcripts of five putative small RNAs (MTBsRNA-

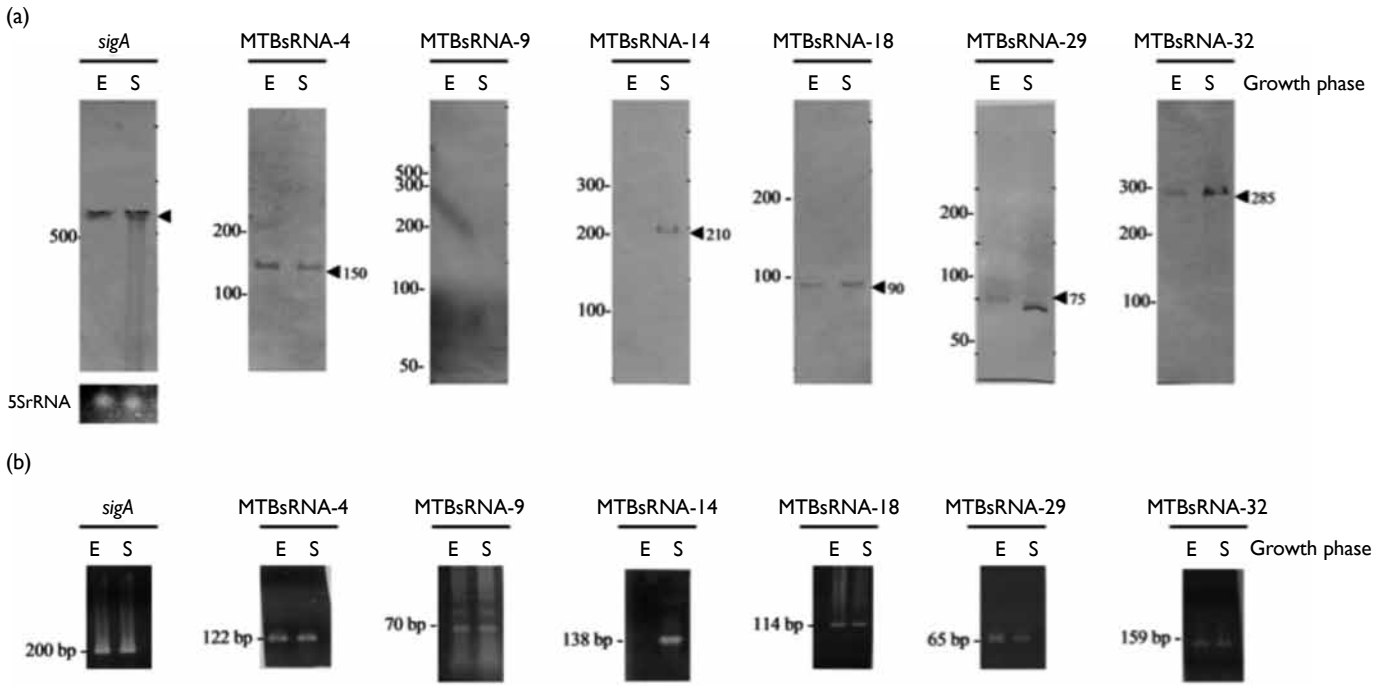

FIG 1. Experimental verification of the predicted small RNAs in different phases of *Mycobacterium tuberculosis* (MTB) growth: MTB cultures grown to exponential (E) or stationary (S) phases were used in this experiment. (a) Northern blots hybridised with digoxigenin-labelled DNA probes. Total RNA samples depleted with rRNAs were separated by denaturing polyacrylamide gel electrophoresis and electro-transferred on to Hybond-N+ membrane, followed by hybridisation with the probes at 42°C overnight. After washing with 2X SSC, the blots were developed for slightly varied time periods; thus the relative intensities of the signals may not accurately reflect the relative abundance of each small RNA transcript. 5S rRNA, which was stained with SYBR Green dye on the gel, was used as a control for RNA loading. The MTB housekeeping gene, sig A, was used as an internal control for monitoring the RNA integrity. Hybridisation bands in the blots are indicated by arrows. The approximate positions of the size standards shown on the left of the blots were obtained by calibrating the blots to a RNA ladder run on a separate gel. The transcript sizes of the small RNAs on the right were estimated by comparing to these standards. For sig A, as the hybridisation band lay outside the range of the standards, its transcript size was not determined. (b) The same batch of RNA samples analysed by northern blotting was used in this RT-PCR analysis. After reverse transcription, the cDNA samples were PCR amplified using the appropriate set of oligonucleotide pairs. After PCR, the reactions were assessed on an agarose gel and stained with SYBR Green dye. The sizes of the reaction products are indicated on the left. The presence of the MTBsRNA-9 transcript was detectable only after RT-PCR amplification.

4, -14, -18, -29, and -32) were detectable by northern analysis (Fig 1a). On the blots, each small RNA was visible as one distinct hybridisation band, suggesting the presence of only one form of transcript for each small RNA under the two growth phases. A comparison of the relative transcript levels of the small RNAs in the two growth phases revealed variation in their expression pattern. For two small RNAs, MTBsRNA-4 and -18, expression appeared to be constitutive as the abundance of their transcripts did not change significantly between the two growth phases. In contrast, up-regulation of expression in the stationary phase was observed for three small RNAs (MTBsRNA-14, 29, and -32). For MTBsRNA-29 and -32, expression in this growth phase was moderately increased. For the small RNA MTBsRNA-14, expression was exclusively induced in stationary phase. As small RNAs are important gene regulators in bacteria, these findings imply a regulatory role of the three differentially expressed MTB small RNAs in mediating gene expression in response to entry into the stationary phase.

The expression of the 47 putative small RNAs in exponential and stationary phases of MTB growth was further examined using RT-PCR (Fig 1b). Judging from the size of the RT-PCR products, transcripts of five small RNAs (MTBsRNA-4, -14, -18, -29, and -32) whose expression had been confirmed by northern blotting were detectable. Moreover, the expression of an additional small RNA (MTBsRNA-9) was also recognised by RT-PCR. Transcripts of this small RNA appeared at an extremely low level in both growth phases as their RT-PCR only showed very faint bands. Detailed analysis of the results from northern blotting and RT-PCR revealed that, for detecting highly expressed small RNAs (eg MTBsRNA-4, -14, -18, -29, and -32), the results from both methods were consistent. Transcripts of those small RNAs detectable in northern blotting were equally detectable by RT-PCR. Nevertheless, for small RNAs expressed at a low level (eg MTBsRNA-9), northern blotting might not be sensitive enough to detect a low level of the transcript. On the other hand, to detect the differential expression of highly expressed small RNA by RT-PCR, owing to its robust amplification efficiency as well as the fact that detection was performed in a background containing a considerable level of the small RNA transcript, RT-PCR may fail to discriminate the expression differences (compare the RT-PCRs of MTBsRNA-14 with MTBsRNA-29 and -32 in Fig 1). Such findings highlight the need to use two complementary methods for a thorough assessment of the expression of the small RNAs.

In summary, this experiment demonstrated the expression of six small RNAs (MTBsRNA-4, -9, -14, -18, -29, and -32). Three of them (MTBsRNA-14, -29, and -32) were shown to express differentially between the exponential and stationary phases of MTB growth and two (MTBsRNA-4 and -18) were shown to express constitutively in both growth phases. The relative expression of MTBsRNA-9 in both growth phases was not clear (Table).

Examination of the effects of oxidative stress on the expression of putative small RNAs in MTB CDC 1551

Bacterial small RNAs are often stress-induced. To investigate how the expression of the 47 putative MTB small RNAs responded to stress similar to that in the host body during the infection process, exponential cultures of CDC 1551 were subjected to oxidative stress (H_2O_2 treatment). Total RNA was isolated and the expression of the 47 small RNA putative small RNAs was examined as described above. For each small RNA, the expression upon stress was compared

TABLE. Expression characteristics of *Mycobacterium tuberculosis* (MTB) CDC 1551 small RNAs identified*

Small RNA	Predicted length (nt)	Approximate observed lengths (nt)	Growth phases				Oxidative stress (H_2O_2)			
			Northern blotting		RT-PCR		Northern blotting		RT-PCR	
			Exponential phase	Stationary phase	Exponential phase	Stationary phase	-	+	-	+
MTBsRNA-4	122	150	√	√	√	√	√	√	√	√
MTBsRNA-9	70	100	×	×	√	√	×	√	√	√
MTBsRNA-14	138	210, 280	×	√	×	√	×	√	×	√
MTBsRNA-18	114	90	√	√	√	√	√	√	√	√
MTBsRNA-24	121	120	×	×	×	×	×	√	×	√
MTBsRNA-29	65	75	√	√√	√	√	√	×	√	×
MTBsRNA-32	159	285	√	√√	√	√	√	√	√	√

* √ and × denote presence and absence of detectable expression, respectively, √√ denotes a higher level of expression, and + and - denote with and without H_2O_2 treatment, respectively

with an unstressed control. Transcripts for a total of seven small RNAs were detectable by both northern blotting and RT-PCR (Fig 2). These included the six small RNAs (MTBsRNA-4, -9, -14, -18, -29, and -32) identified previously and a new small RNA (MTBsRNA-24). It was apparent that oxidative stress induced marked variation in the expression profile of these small RNAs; some remained unchanged (MTBsRNA-4, -18, and -32) and some displayed up-regulation upon stress (MTBsRNA-9, -14, and -24). For the small RNA MTBsRNA-29, expression was down-regulated upon stress. Of particular interest was the small RNA MTBsRNA-14, as over-expression of which was associated with the appearance of a novel transcript (~280 nucleotides), which was not observed in the previous experiment (Fig 1). This finding suggests the presence of two differentially processed transcripts of MTBsRNA-14. Taken together, this experiment further validated the presence of six small RNAs (MTBsRNA-4, -9, -14, -18, -29, and -32) at the transcriptional level as identified in the growth phase experiments, and confirmed the specific expression of an additional small RNA (MTBsRNA-24) under oxidative stress conditions (Table).

Discussion

In this study, up to seven small RNAs were differentially expressed in MTB according to changes in environmental conditions. Production of such sub-cellular elements was up-regulated under adverse conditions such as oxidative stress and those leading to the onset of stationary phase. Identification of small RNAs in MTB and the existence of small RNA-mediated regulatory functions in MTB have been reported,[6,7] but none of the small RNAs identified matched any of the seven MTB small RNAs identified in our study. The number and pattern of actively transcribed small RNA genes in MTB vary between different pathogenic strains and according to changes in environmental conditions. Our work has contributed to a database on the prevalence as well as strain- and condition-specific features of the expression of small RNA genes in MTB. Such database should facilitate future selection of the key elements that play a significant regulatory role in various physiological parameters of this pathogen.

Comparison of the northern blot results and computational predictions of the small RNAs showed inconsistencies in the size of the transcripts, as the actual sizes measurable in our experiments differed by 15-79% from the predicted sizes (Table 1). This may partly be due to estimation of the lengths of small RNAs by northern blotting that may have more than 20% error, as the strong secondary structures can lead to alteration in gel mobility. In addition, the use of the sRNA predicative program, sRNAPredict2, on the GC-rich genome sequence of MTB may result in false predication of the transcriptional endpoints of the putative small RNAs.[4] Taking these factors into consideration, the predicted length and the observed

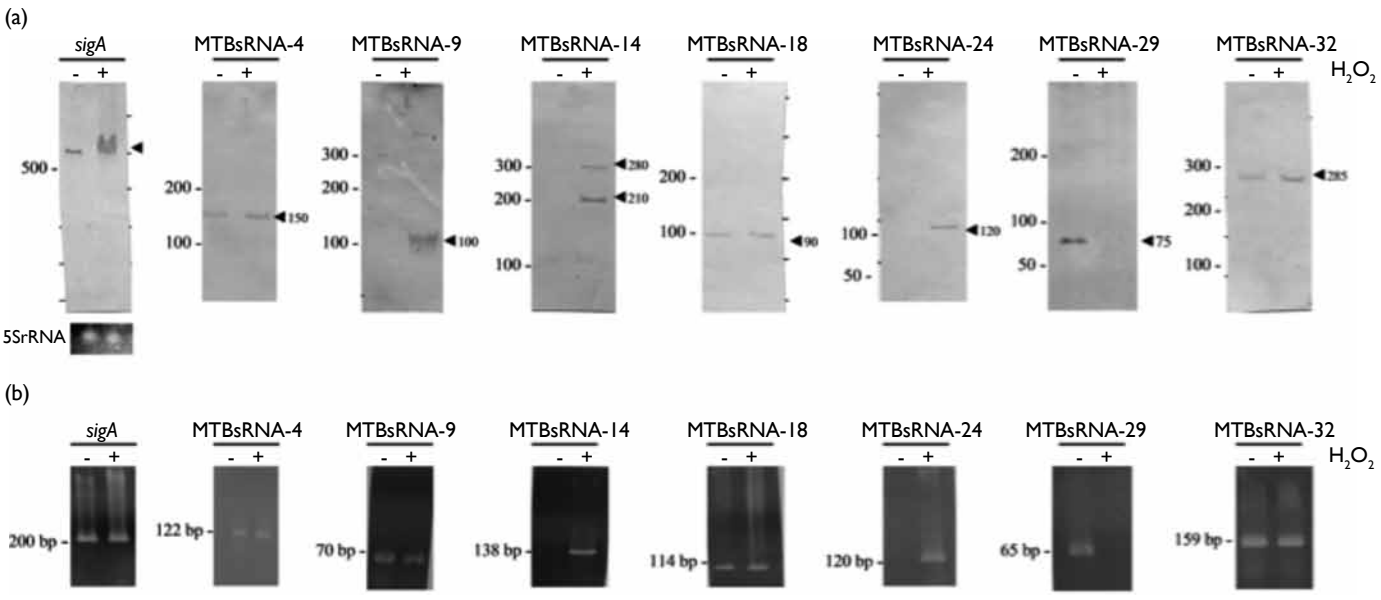

FIG 2. Experimental verification of predicted *Mycobacterium tuberculosis* (MTB) small RNAs after oxidative stress: MTB cells from exponentially grown cultures were collected by centrifugation. After re-suspension in a minimal volume of 7H9 medium, the cells were divided in half. To one half of the cells, ten times the volume of 7H9 medium containing 20 mM of H_2O_2 was added. For the other half, only 7H9 medium was added. After incubating at 37°C for 16 hours, total RNA was isolated from the cells. (a) Northern blot and (b) RT-PCR analyses of RNA samples from the untreated (-) and H_2O_2- treated (+) cells.

length of the small RNAs were within an acceptable range of error in most cases. It is also highly possible that the raw small RNA transcripts are subjected to varying degrees of processing to fine tune their regulatory functions, so that different sizes of small RNA may be generated under different conditions. Future studies should aim to determine the actual size of the transcripts through genome walking, cloning, and nucleotide sequencing, as these experimental approaches can reveal their full genetic sequences. Although sRNAPredict2 predicated correctly 60% of sRNAs for *Pseudomonas*,[7] the rate of predication for MTB was significantly lower, transcripts were detectable only for 7 of the 47 putative sRNAs (about 15%). As sRNAPredict2 predicates sRNAs by searching for co-localisation of genetic structures such as consensus sequences, Rho-independent terminators and putative promoters that are associated with sRNAs in other bacteria, we speculate that sRNAPredict2 may not recognise these genetic structures in the GC-rich genome sequence of MTB. Apart from the difference between the theoretical and measurable size of the small RNA transcripts, a comparison of the results from northern blotting and RT-PCR also revealed discrepancies in these two methods in terms of qualitative difference, level of expression and even product size of such transcripts. For small RNAs whose expression was detectable by northern blotting, the expression was equally recognisable by RT-PCR (eg MTBsRNA-4, -14, -18, -29 and -32). Nonetheless, the reverse was not always true (eg MTBsRNA-9). This phenomenon may be due to the relatively low detection sensitivity of northern blotting. When the expression level of a small RNA is extremely low, such as that of the MTBsRNA-9, the amount of transcript may be well below the detection limit of northern blotting, and hence undetectable. For this reason the expression level of MTBsRNA-9 in both growth phases could not be determined in this study. In addition, for small RNAs expressed at high level (eg MTBsRNA-29 and -32), the RT-PCR reactions appeared to be saturated and failed to discriminate any expression differences. In future studies, we suggest using real-time RT-PCR, which has a higher discriminatory power to test whether the transcription level of specific small RNAs varies according to changes in the level of stress. Furthermore, RT-PCR does not truly reflect the actual size of the transcripts, as the size of the RT-PCR product is defined by the primers, which were designed according to the data generated by theoretical predictions. Hence the size of the transcripts that were measurable in northern blot studies should more accurately reflect the real size of such elements. This explains why the sizes of the transcripts as revealed by northern blotting are mostly larger than the corresponding RT-PCR products.

The small RNA data identified in this study may represent a signature regulatory pattern at the transcriptional level. It is desirable to examine the small RNA expression profile in strains with different virulence levels to assess the correlation between small RNA expression profile and pathogenicity of clinical MTB strains. Together with information on the expression of other virulence factors and proteins responsible for stress response functions, the small RNA data will provide important insight into the basic mechanisms by which MTB maintains long-term viability in the human host and causes infection.

Acknowledgement

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Human epigenetic alterations in *Mycobacterium tuberculosis* infection: a novel platform to eavesdrop interactions between *M tuberculosis* and host immunity

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KEY MESSAGES

1. *Mycobacterium tuberculosis* (MTB) infection altered the methylation levels of inflammatory genes in human macrophages.
2. The induced methylations of the host genes were strain- and host-dependent.
3. The methylation profile of active MTB disease, latent MTB infection, and healthy control groups were distinct. These signatures may potentially be further evaluated as biomarkers in the diagnosis of MTB infection in a clinical setting.
4. The study offers new insights into epigenetic changes in modulating the immune response in MTB infections.

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Introduction

DNA methylation regulates many cellular processes of the human immune system, such as cytokine production, anti-inflammatory response, and cell differentiation. A growing number of diseases and disorders are associated with hypermethylation and hypomethylation of DNA, particularly at the promoter region of functional genes. Functionally, methylation of gene promoter regions impedes the binding of transcriptional proteins and results in transcriptional silencing. Increasing scientific attention has focused on promoter hypermethylation in host cells as a result of bacterial invasion.[1-3]

Mycobacterium tuberculosis (MTB) is an intracellular pathogen that can evade host immunity and survive for a long period of time within human macrophages. Successful bacterial clearance/containment depends upon the activation and appropriate regulation of innate and adaptive immunity pathways, in which macrophages play multiple important roles. TLR-2 activates downstream intracellular signals through MyD88 signalling peptide, leading to the rapid production of cytokines, including IL-1β, in MTB infection. Subsequent macrophage activation and production of pro- and anti-inflammatory cytokines recruit inflammatory cells (T cells, neutrophils, and NK cells) to the area of infection and coordinate the inflammatory and adaptive immune response to MTB.

The epigenetic interaction between MTB infection and host immunity has not been studied. This study aimed to (1) generate the methylation profile of human macrophages before and after infection with MTB so as to correlate MTB infection and methylation alterations, (2) investigate MTB strain dependence on the resulting methylation profiles, and (3) investigate the methylation profiles at different stages of progression of MTB infection by studying macrophages from healthy humans, individuals with latent MTB, and patients with active MTB. The induced hypermethylation could influence the immune response such that MTB intracellular survival and/or pathogenesis is facilitated.

Methods

M tuberculosis H37Rv (ATCC 27294) and 15 clinical isolates of MTB were used: three MDR (INHR, RIFR), three sensitive (INHS, RIFS), three Beijing/W, three non-Beijing/W, and three disseminated strains. The phenotypes and genotypes were respectively confirmed by MIC and DTM-PCR method as described.[4]

Ethics approval was obtained from the Joint CUHK-NTEC Clinical Research Ethics Committee. Whole blood specimens (25 mL) were collected from four healthy controls, three individuals with latent MTB, and three patients with active MTB for monocyte isolation and for QuantiFERON TB-Gold Test (to distinguish between healthy and latent individuals) according to the manufacturer's

instructions.

PBMCs were isolated from 20 mL of whole blood using the Ficoll-Hypaque column. The monocytes were purified from PBMC by removal of detached cells. The monocytes were allowed to differentiate into macrophages for DNA harvesting and downstream experiments.

To induce differentiation to macrophages, 1×10^6 THP-1 cells were first treated with 5 ng/ml PMA. Standard MTB strain H37Rv and the 15 clinical isolates were cultured in Middlebrook 7H9 (BD Biosciences, USA) at 37°C, 5% CO_2 until the cultures reached McFarland 1 (about 10^7 CFU/mL). The MTB cells were harvested. The pellet was re-suspended in RPMI medium, and subsequently added to the macrophages. The macrophages were allowed to engulf the bacilli for 2 hours and the excess free-floating bacilli were removed. The bacterial load in the macrophages was determined by plate counts of the lysed macrophages. The number of intracellular bacilli: macrophage ratio was estimated at 3:1 (range, 2.1 to 3.7 in all subsequent experiments). The RPMI medium was replaced once with fresh medium at 24 hours after infection. Control cultures of THP-1 cells or isolated macrophages were set up with identical corresponding treatments but without MTB infection. DNA was harvested at 72 hours for methylation array study.

The filtered medium from the infected THP-1 differentiated macrophages was analysed for TNF-alpha and IL-1-beta by the Luminex System using the multiplex cytokine assay (Merck Millipore, USA). Results of each cytokine level are expressed as picograms per mL of the filtered medium.

The Human Inflammatory Response Methyl-Profiler DNA Methylation PCR Arrays (SABiosciences, Germany) examined the promoter methylation status of a panel of 24 genes whose involvement in inflammation has been well documented in anti- or pro-inflammatory responses. DNA extracts from macrophages were processed according to the instructions of the manufacturer. DNA was quantified by real-time PCR in each individual enzyme reaction using primers that flanked a promoter (gene) region of interest. The relative fractions of methylated and unmethylated DNA were subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest using the ΔCt method.

Methylated DNA samples from macrophages were extracted following the instructions of the MethylMiner Methylated DNA Enrichment Kit (Applied Biosystems, USA). The Methylated DNA immunoprecipitation–isolated DNA was amplified by GenomePlex Whole Genome Amplification Kit (Sigma, USA) and was then consecutively labelled using the Genomic DNA Enzymatic Labeling Kit. Cy5-labeled methylated DNA and Cy3-labelled input genomic DNA were competitively hybridised onto Agilent Human CpG Island Microarray (consists of 237 220 probes to cover 27 800 CpG island sequences in the human genome), using Oligo aCGH/ChIP-on-chip Hybridization Kit, according to the manufacturer's instructions. The data were normalised to baseline, by dividing Cy5 signal (methylated fragment) by Cy3 signal (total genomic DNA), $\log_2$ transformed and normalised for all the arrays. Data are represented as $\log_2$ (methylated DNA/total genomic DNA) after inter-array normalisation for each probe of the array. Higher $\log_2$ ratios corresponded to higher methylation levels. An ANOVA tool was used to calculate the fold change in methylation for each probe between samples. Signal comparison between macrophages before and after MTB infection, between different strains of MTB, with and without inhibitor treatments, would yield the methylation alteration profiles. Differential methylation with $\log_2 \geq 2$ (~4-fold) signal ratios was used as a cut-off to identify hyper/hypo-methylations.

The relative fractions of methylated and unmethylated DNA were determined using the integrated Excel-based templates downloaded from http://www.sabiosciences.com/dna_methylation_data_analysis.php. The methylated rate was calculated by comparing the amount in each digest with that of a mock (no enzymes added) digest using the ΔCt values of the EpiTect Methyl II PCR Array results. Clustering analysis of methylation levels was performed using online software of the Qiagen website (http://www.sabiosciences.com/dna_methylation_heatmap.php). For the Agilent Human CpG Island Microarray, the array images were digitalised by Agilent Feature Extraction 11.0 (Agilent Technologies, Santa Clara [CA], USA). Data obtained from Feature Extraction were imported to Partek Genomic suite 6.5 for further normalisation and ANOVA computation. The significant features ($P<0.05$) annotated with genes or genes' promoter regions were extracted and imported to Genespring 12.5 for pathway analysis. Comparisons between groups were performed using Mann-Whitney *U* test or Kruskal-Wallis test as appropriate. A P value of ≤0.05 was considered statistically significant.

Results

MTB (H37Rv) infection altered the methylation profile of THP-1 macrophages

H37Rv infection altered the methylation profile of THP-1 macrophages and at 72 hours post-infection: the hypermethylated levels of IL12B, IL4R and the intermedium methylated level of CCL25, IL4R and IL13Ra were noted (Fig 1). The individual promoter methylation levels were compared with those of clinical strains in a later section of the results.

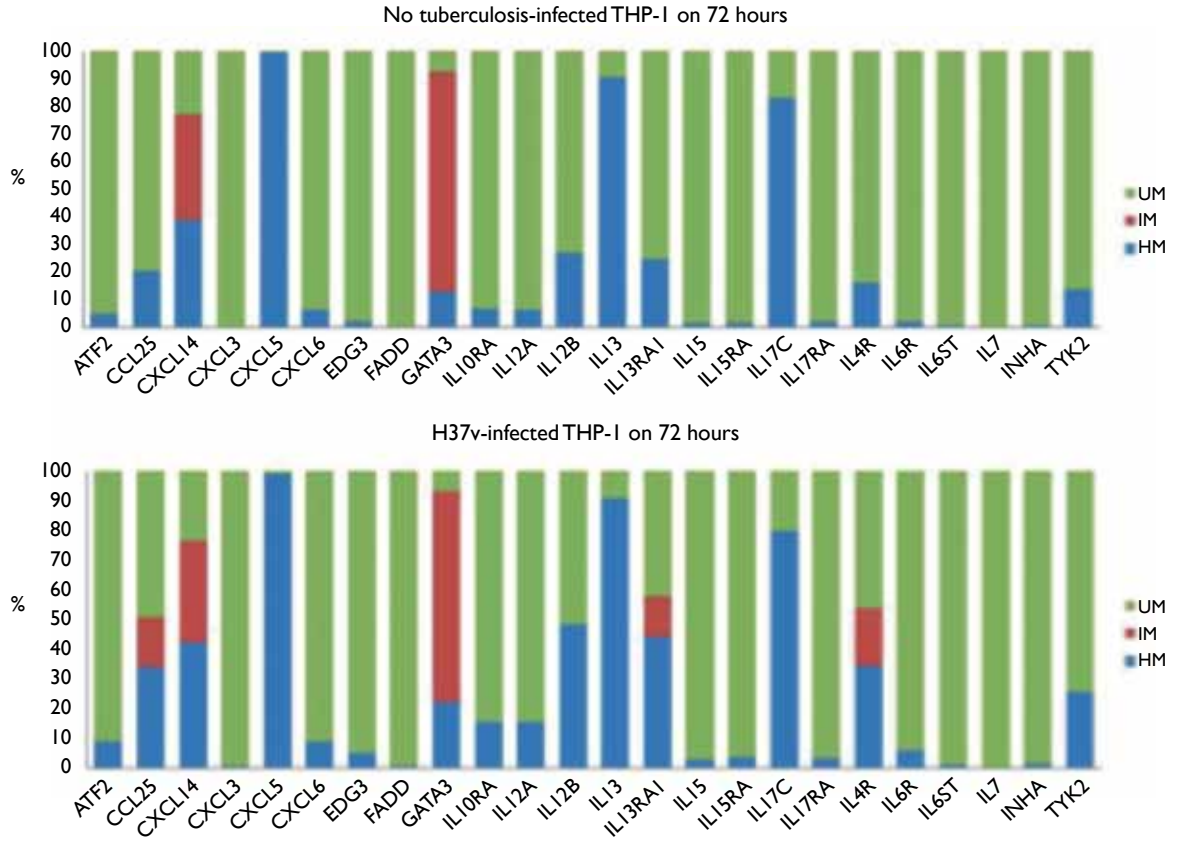

FIG 1. Graphical representation of methylation levels of 24 inflammatory genes in THP -1 macrophages at 72 hours with or without infection with H37Rv*

* Percentages of different methylated fraction of DNA are used to interpret gene methylation status. UM denotes the fraction of input genomic DNA containing no methylated CpG sites, IM intermedium methylated CpG sites, and HM hypermethylated CpG sites in the amplified region of the targeted gene

Methylation profile of different clinical MTB strains on THP-1 macrophages

The level of hypermethylation for each gene from the four groups of strains, namely Beijing/W, non-Beijing/W, strains from disseminated disease, and H37Rv standard strain, were compared. Genes that demonstrated significant difference in methylation levels between these groups are shown in Fig 2. The hypermethylation level of the genes that were significantly different included those of IL17RA ($P<0.027$), IL15RA ($P<0.024$), IL4R ($P<0.024$), IL6R ($P<0.024$), and IL6ST ($P<0.024$) in Beijing/W strains and IL17RA ($P<0.027$), IL6R ($P<0.024$), and IL6ST ($P<0.024$) in non-Beijing/W strains, compared with uninfected macrophages. Only the hypermethylation level of IL4R ($P<0.009$) in THP-1 macrophages showed significant difference in infection with Beijing/W and non-Beijing/W strains. MTB strain from patients with pulmonary disease versus disseminated disease induced significantly higher hypermethylation of IL12A ($P<0.048$) and IL7 ($P<0.048$), and of IL13RA1 ($P<0.048$) in THP-1 infected macrophages. No significant difference was noted between sensitive and resistant MTB isolates (data not shown).

Methylation profile of human macrophages in different patient cohorts

The methylation level in human macrophages of the active MTB disease, latent MTB infection, and healthy control cohorts was examined using Human Inflammatory Response Methyl-Profiler DNA Methylation PCR Array. Significant DNA hypomethylation of FADD ($P<0.023$) and IL17RA ($P<0.049$) was noted in the active MTB disease cohort. Methylated DNA immunoprecipitation in combination with CpG island arrays was performed to characterise at high resolution the DNA

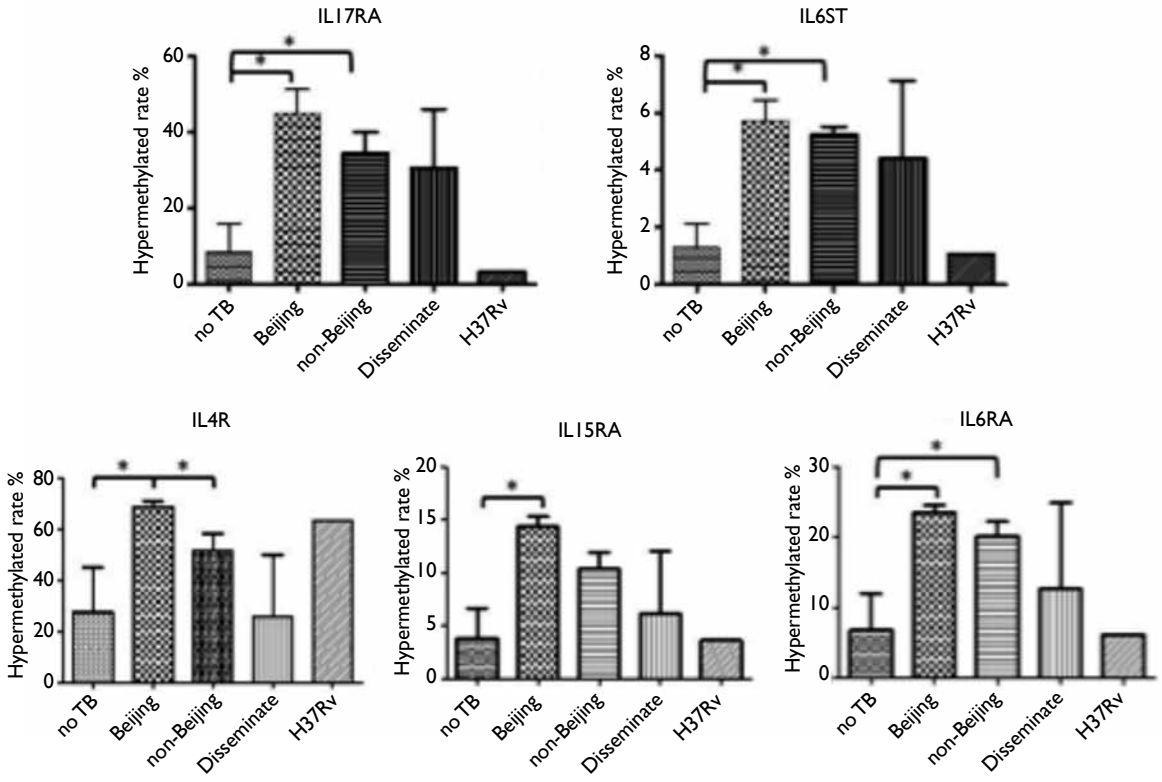

FIG 2. Hypermethylation level in THP-1 macrophages infected with different clinical *Mycobacterium tuberculosis* (MTB) strains, H37Rv-infected, and controls of uninfected THP-1 macrophages. The hypermethylated rate is calculated by comparing the amount in each digest with that of a mock (no enzymes added) digest using the ΔCt values of the EpiTect Methyl II PCR Array

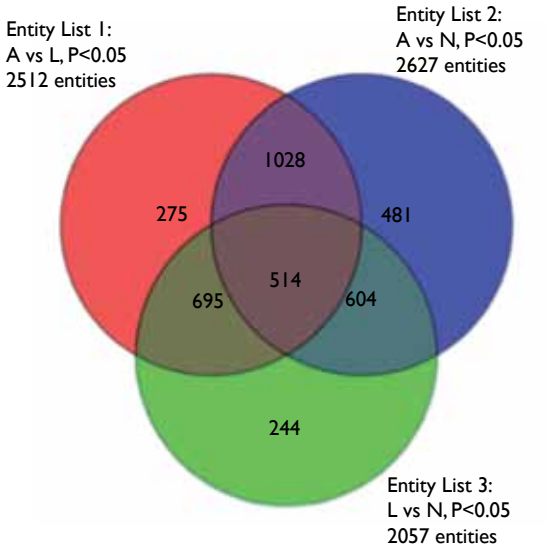

FIG 3. Venn diagram illustrates groups of genes that are differentially methylated when two of the three groups are compared using one-way ANOVA (P<0.05). The red area represented the genes differentially expressed between active and latent *Mycobacterium tuberculosis* (MTB) groups, whereas the blue area between active MTB and healthy control groups and the green area between latent MTB and healthy control groups.

methylation changes that occurred in the genomes of the three cohorts. A Venn diagram illustrates the groups of genes that are differentially methylated comparing two of the three groups (P<0.05, Fig 3). Potentially these may provide specific signatures to distinguish these cohorts for clinical diagnosis.

Discussion

According to the pathway analysis, some biological process in MTB infection was affected by the altered methylation level of different MTB cohorts. Differences between active and latent MTB groups included membrane change through fatty acid and glycan metabolite, signal transduction through GPCR, Notch and Wnt, as well as biological processes such as apoptosis and cell cycle. Changes in the immune response signalling pathway were highly associated with active MTB group, compared with healthy controls. However, difference between the latent MTB group and healthy controls was few. The IL17 signalling pathway was shown to contribute to the inflammatory response against primary MTB infection. MTB infection in active and latent MTB groups could suppress the inflammatory reaction by enhancing the hypermethylated level of IL17RA

and downregulating that of IL17D, compared with healthy controls. Only the hypermethylated level of IL17RD and IL17C was differentially expressed between the active and latent MTB groups. The mechanisms of methylation in IL17 family associated with MTB infection warrant further study.

IL6 plays a key role in MTB infection. MTB regulates host IL6 production to inhibit type I interferon signalling. MTB infection induced IL6R promoter partial methylation in the macrophages, but how the methylated epigenomic modification affects IL6R expression and function remains elusive. In addition, IL-6 is one of the major cytokines responsible for differentiating T-helper lymphocytes into Th17 cells, further confirming the interrelationship and importance of these two pathways in MTB infection.

DNA methylation has been the most studied epigenetic alteration and an important biomarker in cancer diagnosis, but little is known about its role in MTB infection. To investigate DNA methylation changes associated with MTB infection, the common methylation profile of infection with different clinical MTB strains in different MTB hosts was studied. The results provide insight into the molecular mechanisms and biological pathways that underlie MTB infection and correlate CpG island methylation status with anti- or pro-inflammatory responses.

Our data confirm the occurrence of DNA hypermethylation of inflammatory genes in different clinical MTB strains and MTB groups, and these profiles may be biosignatures that signify latency or disease. Our studies also lead to the discovery of novel methylated genes that could be implicated in MTB infection.

The hypothesis that MTB infection can alter the methylation profile of the host cells is proven. The methylation profiles of varying factors, including the duration of infection, strain type, and host type were obtained. Our study proved that the induced methylation profiles are strain-dependent and host-dependent. The resulting methylation-profiles will enhance our understanding of MTB and human immunity, substantiate potential strain-dependence and host-dependence on the outcome of infection, and aid future drug design. There is potential application of the methylation profile as a biomarker in the diagnosis of MTB infections.

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Prevalence of carriage and characterisation of methicillin-resistant *Staphylococcus aureus* in slaughter pigs and personnel exposed to pork carcasses

MV Boost *, M O'Donoghue, L Guardabassi

KEY MESSAGES

1. There is a high level of contamination of pig carcasses in Hong Kong with multi-drug resistant methicillin-resistant *Staphylococcus aureus* (MRSA) ST9.
2. Personnel occupationally exposed to pigs and pig carcasses are at risk of colonisation and possible infection.
3. Whilst the clinical significance of porcine MRSA ST9 is unclear, there is a need for continued surveillance of this potential reservoir of MRSA.
4. Butchers should be encouraged to wear gloves whilst working and maintain good personal hygiene to reduce colonisation risk.

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Introduction

In recent years, methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged in the community. There have been increasing reports of MRSA colonisation of pigs and pig farmers in Europe[1] and North America. Multi-locus sequence typing (MLST) revealed widespread dissemination of a particular sequence type (ST) 398 among pigs in the Netherlands and subsequently the occurrence of this MRSA lineage has been reported in several other countries and animal species. Compelling microbiological and epidemiological evidence indicates that people who live or work on farms, especially pig farms, have an increased risk of colonisation or infection with ST398 MRSA. Human infection with ST398 MRSA has been reported in Hong Kong, but no information about occupation or pig contact was available.

Porcine-associated MRSA ST398 typically harbours Staphylococcal cassette chromosome (SCC) *mec* type IV or V, but unlike most community MRSA, ST398 is increasingly multi-drug resistant. It appears that the genes for Panton-Valentine leucocidin toxin are rare in these strains. ST398 is non-typable by pulsed field gel electrophoresis (PFGE) using the restriction endonuclease *sma*1, which is commonly used in the investigation of clonality of staphylococcal strains.

This study aimed to (1) determine the MRSA colonisation level of locally slaughtered pigs and whether this colonisation results in spread to persons occupationally exposed, (2) characterise any MRSA isolates for the presence of toxin mediating genes, and (3) compare ST398 MRSA strain in this locality with the predominant strain reported elsewhere.

Methods

This study was conducted from January 2009 to December 2012. Ethics approval was obtained from the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University. Nasal samples were collected from 400 slaughtered pigs after distribution to three wet markets in Hong Kong over a 3-month period. Butchers at all major wet markets in Hong Kong were invited to participate. A total of 300 butchers consented to sampling by self-collection of a nasal swab according to instructions. Butchers were asked about any recent hospitalisation, healthcare workers in the family, wound infections within the last year, and antibiotic use in the last 6 months. Nasal swabs were collected from 100 to 150 pigs of different ages (weaning pigs, market ready animals, and breeding sows) from two farms each in Hong Kong (n=220) and Guangdong (n=255). A small number of environmental samples were collected at the Hong Kong farms.

Swabs were enriched in brain heart infusion, cultured on selective agar and presumptive MRSA colonies were identified as *S aureus* by latex agglutination. Susceptibility to a range of antibiotics was determined following standard guidelines.

MRSA isolates were tested by PCR for the

*mec*A gene, resistance determinants for macrolides, tetracycline, and chloramphenicol and subjected to SCC*mec* typing and staphylococcal protein A gene (*spa*) sequencing (http://spaserver.ridom.de). All isolates were screened for genes for Panton-Valentine leucocidin, enterotoxins (*sea, see*), and exfoliative toxins. PFGE using *sma*1 digestion was performed on one representative sample from each of the antibiotic susceptibility patterns. Isolates representative of a distinct PFGE pattern and those of non-t899 spa types were analysed by MLST.

Results

MRSA was detected in 39.3% (95% confidence interval [CI]=38.8-39.8%) of slaughtered pigs and overall 170 MRSA strains were isolated and confirmed to harbour *mec*A, SCC*mec* types IVb (92%) or V (8%), and belong to *spa* type t899 or closely related variants; t4474 (2 isolates), and one isolate each of t1939, t2922, and t5390.

Seventeen (5.7%, 95% CI=4.2-7.0%) butchers were MRSA-colonised. Fifteen strains harboured SCC*mec* IV of which ten were t899 and belonged to ST9. The remaining type IV strains were t008 (ST8), t002 (ST5), and t123 (ST45), all of which have been reported from skin and soft tissue infections in Hong Kong, and single isolates of t359 (ST747) and t375 have been reported from buffaloes and nasal colonisation, respectively. Two strains were SCC*mec* II. These were healthcare-associated t701 (ST6) and were isolated from two workers at the same stall. None of the butchers had been recently hospitalised nor had a healthcare worker in their immediate family. Two of those colonised had received antibiotics in the last 6 months, one for a skin infection, and four reported a wound infection within the last year. All butchers were exposed to meat for at least 9 hours per day and none routinely wore gloves.

None of the samples collected at the Hong Kong farms yielded MRSA, but a high percentage of pigs at both mainland farms were colonised. The rate of colonisation of the pigs varied considerably between farms (Table 1). At farm 1 all isolates were t899 (ST9), but at farm 2 three isolates had *spa* types closely related to t899; t1334 (2 isolates) and t4358.

The majority of carcass isolates were multi-drug resistant (Table 2); 91% were resistant to four or more classes of non-beta-lactam antibiotics. The most predominant pattern (45% of isolates) included resistance to fluoroquinolones, tetracyclines, macrolides/lincosamides/streptogramins, and chloramphenicol. Resistance to fusidic acid and rifampicin was uncommon. ST9 isolates from butchers were much more resistant than non-livestock-associated strains (90% resistant to clindamycin, 80% resistant to chloramphenicol, quinupristin/dalfopristin, erythromycin, and tetracycline, 50% resistant to ciprofloxacin, 30% to fusidic acid, and 20% to cotrimoxazole and gentamicin). Resistance rates were quite similar in isolates from the two mainland farms although farm 2 was lower in terms of some agents (Table 3). Overall no isolates were resistant to linezolid, nitrofurantoin, or tigecycline.

All resistance to erythromycin was attributable to *erm*(C), and chloramphenicol resistance to *fex*(A). No chloramphenicol-resistant isolate was positive for *cfr* and there was no resistance to linezolid although MICs were close to the resistance breakpoint. All tetracycline-resistant strains carried *tet*(K), but 3% additionally carried *tet*(M). No virulence factor genes were detected. There was no relationship between SCC*mec* type and occurrence of antimicrobial resistance genes in pig isolates, but in human isolates there was more variation with some SCC*mec* type IV and V isolates displaying limited resistance, characteristic of typical community MRSA strains, whereas others were t899 and matched the pig isolates. Isolates found to be type II were resistant only to the beta-lactams.

PFGE of representative strains of carcass isolates revealed eleven different banding patterns by *sma*1digestion (Table 2). Cleavage of the DNA by *sma*1 suggested that the strain was not ST398. MLST subsequently confirmed that all isolates were ST9. The banding patterns showed that many of the strains were closely related, and this relationship was associated with the SCC*mec* type. PFGE of strains from live pigs revealed related strains. The banding patterns displayed by the human isolates varied much more, but those of t899 closely matched the porcine strains.

Discussion

Samples from the anterior nares of slaughtered pigs at three representative regional wet markets in Hong Kong demonstrated a high MRSA colonisation rate, which was comparable with the situation in the Netherlands and Belgium (~40%).[1] The colonisation rate in butchers was much higher than that reported for the general public in Hong Kong (~1%) and exceeded that of healthcare workers (3.2%). Of the

TABLE 1. Methicillin-resistant *Staphylococcus aureus* (MRSA) colonisation of live pigs at farms in Guangdong

Pig type	% (No. of MRSA positive/total No. sampled)	
	Farm 1	Farm 2
Weaners	73.5 (25/34)	34 (17/50)
Finishers	75.8 (25/33)	38 (19/50)
Sows	0 (0/38)	44 (22/50)
Total	47.6 (50/105)	38.6 (58/150)

TABLE 2. Susceptibility patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) strains (n=170) isolated from pig carcasses

No. of strains	Market			Antimicrobial resistance*									SCC*mec* type	PFGE type	*spa* type
	Ngau Chi Wan (n=100)	Shatin Central (n=100)	Hung Hom (n=200)	C	Cip	Da	E	F	QD	R	Sxt	T			
37	5	5	27	+	+	+	+		+			+†	IVb	A	**t899**
26	0	6	20	+	+	+	+					+	IVb	A1	t899
14	1	5	8		+	+	+					+†	IVb	A2	t899
12	4	3	5	+	+	+	+		+		+	+†	IVb	A3	t899
10	5	1	4	+		+	+		+			+	V	F1	t1939
9	1	3	5	+	+	+	+				+	+	IVb	A1	t2922
9	0	4	5		+	+	+				+	+	V	F	t5390
7	1	3	3	+		+	+				+	+	IVb	A	t899
6	3	0	3		+	+	+		+			+	IVb	B	t4474, t899
4	0	2	2			+	+					+	IVb	A	t899
3	1	2	0			+						+	IVb	B	t899
3	1	0	2	+		+	+					+	IVb	A	t899
2	0	0	2		+	+						+	IVb	A3	t899
2	2	0	0	+	+	+		+			+	+	IVb	A	t899
2	1	0	1		+	+	+		+		+	+	IVb	B1	t4474
2	0	0	2	+	+	+						+	IVb	A	t899
2	0	1	1			+	+		+		+	+	IVb	A	t899
2	0	1	1	+		+						+	IVb	A	t899
2	0	1	1	+	+	+					+	+	IVb	A	t899
1	1	0	0		+							+	IVb	E	t899
1	0	0	1			+		+			+		IVb	A	t899
1	0	0	1			+		+				+	IVb	A	t899
1	0	1	0	+	+	+		+		+	+	+	IVb	A	t899
1	0	0	1	+	+	+	+	+			+	+	IVb	A	t899
1	0	0	1		+	+			+		+	+	IVb	A	t899
1	0	1	0	+	+	+	+		+		+		IVb	A1	t899
1	0	1	0		+	+	+				+		IVb	A	t899
1	1	0	0	+	+	+	+		+	+		+	IVb	A	t899
1	0	1	0	+	+	+	+			+		+	IVb	A	t899
1	1	0	0	+	+	+	+			+	+	+	IVb	A	t899
1	0	1	0	+	+	+	+						IVb	D	t899
1	0	0	1	+		+	+		+		+	+	IVb	A	t899
1	0	1	0			+	+				+	+	IVb	A	t899
1	0	1	0			+	+				+		V	C	t899
1	0	0	1			+	+		+			+	IVb	A	t899
No. (%) of MRSA strains resistant	28 (28)	44 (44)	98 (49)	120 (71)	133 (78)	169 (99)	152 (89)	6 (4)	74 (44)	4 (2)	55 (32)	165 (97)			

* C denotes chloramphenicol, Cip ciprofloxacin, Da clindamycin, E erythromycin, F fusidic acid, QD quinupristin-dalfopristin, R rifampicin, Sxt co-trimoxazole, and T tetracycline. No strains were resistant to vancomycin, linezolid, nitrofurantoin, or tigecycline

† *tet* (M) was found in strains of these three groups (3/37, 1/14, and 1/12, respectively)

colonised butchers, the highest proportion carried ST9 (3.3%). It was only possible to sample these workers on one occasion; it would be useful to determine whether the colonisation was persistent. Persistence of ST398 colonisation in farmers depends on the intensity of contact.[2] All our subjects were exposed to meat for a minimum of 9 hours daily, with many working 7 days each week. Such

TABLE 3. Percentage resistance to antibiotics of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from pigs at two farms in Guangdong

Farm	Antimicrobial resistance* (%)								
	C	Cip	Da	E	F	QD	CN	Sxt	T
1	44	84	100	98	7	91	49	3.5	96.5
2	32	100	100	100	5	57	25	5	93

* C denotes chloramphenicol, Cip ciprofloxacin, Da clindamycin, E erythromycin, F fusidic acid, QD quinupristin-dalfopristin, CN gentamicin, Sxt co-trimoxazole, and T tetracycline

levels of colonisation would pose an increased risk of MRSA infection.

Interestingly, MRSA was not isolated from pigs or environmental samples at either of the Hong Kong pig farms with 3000-5000 pigs and 3-4 employees. The pig accommodation was covered but not enclosed, allowing constant fresh air circulation. Both farms claimed to use no antibiotics except for individual sick animals that would be isolated during illness. Unfortunately, we were unable to gain access to any other farms in Hong Kong, so the absence of MRSA from all farms cannot be assumed. In mainland China, both farms visited were very large and comprised several enclosed barns with fan-assisted ventilation. The infection control precautions were strictly enforced. Nevertheless, MRSA was present on both farms at levels similar to that found for MRSA ST398-infected farms in the Netherlands (42%). The managers claimed that the pigs did not receive antibiotic-containing feed. Use of prophylactic antibiotics could not be confirmed, but use for sick animals was reported.

At the commencement of this study, it was expected that any porcine MRSA detected would most likely be ST398, the predominant porcine strain reported in Europe and North America. Nonetheless, although the isolates were t899, they were typable by PFGE with *sma*1. MLST revealed that the strains were ST9. Other researchers have reported the presence of MRSA ST9 on farms in mainland China, although their sampling was performed in different regions.[3]

Twelve animals from one of the Hong Kong farms were tracked through the slaughtering and butchering processes. Although negative before commencing their journey to the abattoir, they were all positive for MRSA after slaughter, suggesting cross-infection at the central abattoir. We requested to be allowed to sample in the abattoir but were denied access.

In contrast with European swine MRSA isolates in which SCC*mec* III, IVa, and V have been reported, only SCC*mec* IVb and V were present in our porcine isolates. This suggests relatively limited genetic diversity among Asian swine MRSA isolates, and horizontal transfer is not an important mechanism of transfer between strains. Although SCC*mec* types IV and V are typical of community MRSA, our ST9 strains were resistant to multiple classes of antibiotics, which is unusual in community MRSA. The high degree of resistance in porcine isolates suggests wide exposure to antibiotics, possibly used in pig husbandry. The major source of pigs for the Hong Kong market is Mainland China. Such multi-resistance to antibiotics used in human medicine could make infection with swine MRSA difficult to treat.

Tetracycline resistance in porcine isolates was mainly mediated by *tet*(K). Only a few porcine isolates harboured two tetracycline resistance determinants. In contrast, the majority of ST398 isolates from Germany harboured two determinants. Erythromycin resistance in porcine strains was mediated by *erm*(C). This finding is similar to that reported in Europe, although *erm*(A) and *erm*(B) have been reported in Germany. All human ST9 isolates also harboured *erm*(C).

Although virulence factors are not commonly found in swine MRSA, the high colonisation rate of pigs indicates a need for continued surveillance of this potentially large reservoir for human infections. This study has shown the potential for ST9 porcine strains to colonise humans exposed to pork meat. In addition, there is a need to look at possible transmission of these strains to humans via the food chain and via contact with persons working with pigs.

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Knowledge, attitude, and behaviour toward antibiotics among Hong Kong people: local-born versus immigrants

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KEY MESSAGES

1. The general public's knowledge about antibiotics is inadequate.
2. 30% of the public would expect or request antibiotics for a common cold, but 40% would not complete the full course.
3. <40% of the public thought that they could help prevent antibiotic resistance.
4. Age and education were the main determinants of knowledge, attitude, and behaviour toward antibiotics.
5. New immigrants did not differ from the local-born except that they were more likely to buy antibiotics over the counter and to keep the left-over.

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Introduction

The World Health Organization marked "Antimicrobial resistance: no action today, no cure tomorrow" as the theme of World Health Day 2011. Antimicrobial resistance (AMR) is an urgent global threat.[1] The misuse of antibiotics for upper respiratory tract infections (URTI) is a main cause of AMR. Local studies showed that 237 (23.7%) of 1002 interviewed citizens were prescribed antibiotics for their last URTI[2]; 27.5% of consultations for URTI ended with antibiotics[3]; and 33.9% of primary care doctors prescribed antibiotics for >40% of URTI consultations.[4] Some doctors reported that their patients expected antibiotics,[4] and at least 26% of patients required antibiotics when they consulted doctors for URTI.[2]

Patient knowledge, attitude, and behaviour toward the use of antibiotics might not only affect whether an antibiotic is prescribed appropriately but also how it is consumed. Cultural and economic factors also affect antibiotic use.[5] There was one such study in Hong Kong, but it was limited by the small number (n=12) of questions, a restrictive scoring system, and a 14% response rate.[2]

We aimed to investigate the public's knowledge, attitude, and behaviour toward antibiotics in both local-born and immigrant populations.

Methods

This combined qualitative and quantitative study was conducted from August 2009 to January 2011. The former comprised focus groups and in-depth interviews with purposively sampled participants. The latter comprised a territory-wide telephone survey.

Members of 236 social centres in Hong Kong were invited to participate. Eight focus groups with six to eight participants each were conducted. One of the groups consisted of eight new immigrants who had stayed in Hong Kong for about 5 years. An experienced facilitator and an investigator experienced in qualitative research conducted the discussions. Discussions were conducted in Cantonese, audio-taped, and transcribed verbatim. Thematic analysis was used to interpret the transcripts. Two independent investigators read and coded all the transcripts separately, and the two investigators jointly abstracted themes from the coded texts. The quotations below were translated into English for this report.

A questionnaire was developed based on the data collected from focus groups. It was then pilot tested on 50 successfully completed telephone interviews; these data were excluded from analysis.

The Social Sciences Research Centre, the University of Hong Kong, conducted the survey between 6 pm and 10 pm on weekdays from November to December 2010. All interviewers were trained to conduct the questionnaire and completed a practice interview before making phone calls. The target population was randomly selected Cantonese-speaking residents aged 18 years or over in Hong Kong. When contact was successfully established

with a target household, an adult with the nearest next birthday was selected, excluding persons with communication difficulties. A maximum of five attempts were made for unanswered lines.

Of all immigrants, a 'new immigrants' subset was specified for those who had stayed in Hong Kong for ≤10 years. Those born in Hong Kong were compared with all immigrants, and with the new immigrants.

A 'half-half' proportion from the respondents was assumed. To ensure that the error would be at most 0.02 with 95% confidence, a sample size of 2401 was required. For binary or ordinal responses, multiple logistic or ordinal regression analysis was used to identify the risk factors associated with the respondent's choice. Pearson chi-squared test was used to determine whether nominal responses were dependent. The Student's *t*-test was used to compare interval responses between groups.

Results

A total of 21 males and 35 females aged 20 to 73 years took part in eight focus groups (6-8 per group). Of these, 28.8% had completed tertiary education, 46.2% secondary, and 25.0% primary or below. Two males and two females aged 33 to 61 years participated in the in-depth interviews.

Of 3996 successful calls to households, 157 calls had language problems and 219 were not qualified. Of the remaining 3620 calls, 813 refused to be interviewed, 336 did not complete the interview, and 2471 (864 males and 1607 females) completed the interview (response rate, 68.3%). The age distribution was comparable with the Hong Kong population in the 2010 By-census. Of them, 1634 (66.1%) were born in Hong Kong, 729 (29.5%) in Mainland China, 88 (3.6%) elsewhere, and 20 (0.8%) refused to answer. The local-born and immigrants were comparable in terms of gender distribution (male:female ratio was 584:1050 and 275:542 respectively, χ^2=1.92, P=0.38).

The immigrants had stayed in Hong Kong for 31.8±17.99 (median, 30) years. The mean years of stay was 14.4±8.18 for young adults (age <40 years), 30.76±14.40 for older adults (age 40-64 years), and 49.1±14.35 for the elderly (age ≥65 years). There were more elderly and fewer with tertiary education among the immigrants (P<0.001, Pearson Chi-squared test, Table 1). Young adults were more likely to have a higher education (χ^2=520.31, P<0.001).

Of all the immigrants from mainland China, 134 were new immigrants. Their mean years in Hong Kong was 7.4±2.74 years. Relative to the local-born, the new immigrants were younger (Wilcoxon V=325, P<0.001) and of a higher proportion of female (χ^2=26.460, P<0.001) and secondary education (χ^2=27.223, P<0.001).

Knowledge

Focus-group participants were uncertain about terms such as drug resistance, anti-inflammatory drugs, effectiveness against viral infections like URTI, and the side-effects. Those with a higher education or new immigrants did not differ to others. Some exemplary quotes were:

"Killing bacteria and anti-inflammation." (FG4.P1_p1)

"…I perceive antibiotics are like those medicine with strong potency." (FG2.P1_p1)

"I really have no idea." (FG3.P1_p28)

In the telephone survey, immigrants were more likely to give the response 'don't know' (Table 2). The local-born and immigrants did not significantly differ

TABLE 1. Distribution of age and education groups among telephone survey participants

Age and education	No. (%) of participants		
	Local-born (n=1602)	All immigrants (n=803)	New immigrants (subgroup of all immigrants) [n=131]
Age <40 years			
Primary education or below	6 (0.4)	6 (0.7)	2 (1.5)
Secondary education	218 (13.6)	109 (13.6)	57 (43.5)
Tertiary education	306 (19.1)	72 (9.0)	18 (13.7)
Age 40-64 years			
Primary education or below	151 (9.4)	106 (13.2)	5 (3.8)
Secondary education	567 (35.4)	239 (29.8)	39 (29.8)
Tertiary education	251 (15.7)	58 (7.2)	8 (6.1)
Age ≥65 years			
Primary education or below	54 (3.4)	110 (13.7)	0
Secondary education	37 (2.3)	75 (9.3)	1 (0.8)
Tertiary education	12 (0.7)	28 (3.5)	1 (0.8)

TABLE 2. Responses to questions on knowledge

Question	No. (%) of participants			χ^2	P value
	Local-born	All immigrants	Total sample		
Know why antibiotics prescribed				44.138*	<0.001
Yes	997 (61.1)	404 (49.5)	1401 (57.3)		
No	551 (33.8)	323 (39.6)	874 (35.7)		
Don't know	83 (5.1)	89 (10.9)	172 (7.0)		
Different antibiotics for different infections				2.519	0.284
Yes	1380 (84.5)	677 (82.9)	2057 (83.9)		
No	80 (4.9)	36 (4.4)	116 (4.7)		
Don't know	174 (10.6)	104 (12.7)	278 (11.3)		
Effective for bacteria				37.029*	<0.001
Yes	1208 (73.9)	550 (67.3)	1758 (71.7)		
No	126 (7.7)	36 (4.4)	162 (6.6)		
Don't know	300 (18.4)	231 (28.3)	531 (21.7)		
Effective for virus				39.491*	<0.001
Yes	926 (56.7)	413 (50.6)	1339 (54.6)		
No	269 (16.5)	89 (10.9)	358 (14.6)		
Don't know	439 (26.9)	315 (38.6)	754 (30.8)		
Effective for common cold				36.242	<0.001
Yes	774 (47.4)	331 (40.5)	1105 (45.1)		
No	560 (34.3)	249 (30.5)	809 (33.0)		
Don't know	300 (18.4)	237 (29.0)	537 (21.9)		
Effective for inflamed throat				42.365	<0.001
Yes	888 (54.3)	409 (50.1)	1297 (52.9)		
No	456 (27.9)	173 (21.2)	629 (25.7)		
Don't know	290 (17.7)	235 (28.8)	525 (21.4)		
Effective for urinary tract infection				35.323	<0.001
Yes	949 (58.1)	377 (46.1)	1326 (54.1)		
No	146 (8.9)	73 (8.9)	219 (8.9)		
Don't know	539 (33.0)	367 (44.9)	906 (37.0)		
Undesirable to stop early when symptom-free				81.874*	<0.001
Yes	1136 (69.5)	453 (55.4)	1589 (64.8)		
No	343 (21.0)	182 (22.3)	525 (21.4)		
Don't know	155 (9.5)	182 (22.3)	337 (13.8)		
Undesirable to purchase over the counter				34.779	<0.001
Yes	1141 (69.6)	496 (60.7)	1637 (66.8)		
No	314 (19.2)	163 (20.0)	477 (19.5)		
Don't know	179 (11.0)	158 (19.3)	337 (13.7)		
Antibiotics have possible side-effects				35.540*	<0.001
Yes	1197 (73.3)	590 (72.2)	1787 (72.9)		
No	238 (14.6)	69 (8.4)	307 (12.5)		
Don't know	199 (12.2)	158 (19.3)	357 (14.6)		
Drowsiness as a side-effect				18.601*†	<0.001
Yes	494 (41.3)	255 (43.2)	749 (41.9)		
No	591 (49.4)	244 (41.4)	835 (46.7)		
Don't know	112 (9.4)	91 (15.4)	203 (11.4)		

* Remains significant after exclusion of 'don't know'

† Only interviewees who agreed with possible side-effects from antibiotics were analysed

‡ Only interviewees who always finished the full course of antibiotics were analysed

TABLE 2. Cont'd

Question	No. (%) of participants			χ^2	P value
	Local-born	All immigrants	Total sample		
Loss of appetite as a side-effect				28.476*†	<0.001
Yes	507 (42.4)	280 (47.5)	787 (44.0)		
No	557 (46.5)	205 (34.7)	762 (42.6)		
Don't know	133 (11.1)	105 (17.8)	238 (13.3)		
Sweating as a side-effect				21.129*†	<0.001
Yes	382 (31.9)	240 (40.7)	622 (34.8)		
No	592 (49.5)	225 (38.1)	817 (45.7)		
Don't know	223 (18.6)	125 (21.2)	348 (19.5)		
Only full course to be effective				1.868‡	0.393
Yes	913 (85.2)	383 (82.9)	1296 (84.5)		
No	111 (10.4)	52 (11.3)	163 (10.6)		
Don't know	47 (4.4)	27 (5.8)	74 (4.8)		
May be ineffective next time if not full course				3.473‡	0.176
Yes	652 (60.9)	274 (59.3)	926 (60.4)		
No	272 (25.4)	108 (23.4)	380 (24.8)		
Don't know	147 (13.7)	80 (17.3)	227 (14.8)		
Duration of common cold				14.870*	0.005
1-3 days	381 (23.3)	162 (19.8)	543 (22.2)		
4-6 days	419 (25.6)	189 (23.1)	608 (24.8)		
1-2 weeks	756 (46.3)	401 (49.1)	1157 (47.2)		
>2 weeks	37 (2.3)	29 (3.5)	66 (2.7)		
Don't know	41 (2.5)	36 (4.4)	77 (3.1)		

for questions: (1) different antibiotics for different infections, (2) only full course to be effective, and (3) incomplete course leading to ineffectiveness next time. The local-born were more likely to give the correct response except for questions: (1) antibiotics having possible side-effects and (2) expected duration of a common cold. Of all interviewees, 223 (9.0%) were not familiar with the term 'drug-resistance'.

One mark was given for a correct response to each of 16 questions; the mean total score was 7.8±3.14. The local-born scored better than all immigrants (8.1±3.02 vs 7.2±3.28, t=7.241, P<0.001), but the subset of new-immigrants scored similarly to the local-born (7.7±2.78 vs 8.1±3.02, t=1.64, P=0.10).

Attitude

Many focus-group participants trusted the doctor's decision on whether to prescribe antibiotics. However, some participants in the new-immigrant group had different ideas:

> "When you get sick and have to consult a doctor, you have to trust the doctor. When the doctor's prescription requires you to finish all the medicine, you have to finish it all…." (FG1.P2_p22)

> "He had a casual look [at my son] and then decided to prescribe an antibiotic. Hence I refused." (FG7.P8_p25)

> "Prescribing [antibiotics] for colds and flu is not necessary. For doctors, you should prescribe antibiotics only with inflammation, with bacteria; otherwise, you should not." (FG7.P2_p19)

The focus-group participants generally opined that the doctors and the government were the main groups responsible for the prevention of antibiotic resistance.

> "I think, the doctor has the greatest responsibility [for antibiotic abuse]." (FG5.P1_p19)

> "I gather that there should be two aspects that can be worked on. First is the doctors' integrity…. Second, the government's education department…should be very important." (FG8.P4_p20)

In the telephone survey, both the local-born and immigrants agreed that fewer courses of antibiotics would diminish drug resistance (Table

TABLE 3. Responses to questions on attitude

Question	No. (%) of participants			χ^2	P value
	Local-born	All immigrants	Total sample		
Taking more courses of antibiotics would weaken immunity				7.567	0.006
Agree	1267 (85.4)	617 (89.7)	1884 (86.7)		
Disagree	217 (14.6)	71 (10.3)	288 (13.33)		
Types of doctor's antibiotics-prescribing behaviour preferred				10.996	0.012
Rarely	631 (41.7)	264 (37.1)	895 (40.2)		
Readily	25 (1.7)	21 (2.9)	46 (2.1)		
On request	69 (4.6)	48 (6.7)	117 (5.3)		
Indifferent	790 (52.1)	379 (53.2)	1169 (52.5)		
Fewer courses of antibiotics taken would lead to less drug resistance				0.096*	0.818
Agree	1214 (88.6)	547 (89.1)	1761 (88.8)		
Disagree	156 (11.4)	67 (10.9)	223 (11.2)		
Fewer prescriptions by doctors would lead to less drug resistance				0.13*	0.72
Agree	1139 (85.4)	503 (84.8)	1642 (85.3)		
Disagree	194 (14.6)	90 (15.2)	284 (14.7)		
Antibiotic resistance was a serious problem in Hong Kong				8.17*	0.004
Agree	887 (72.9)	402 (79.4)	1289 (74.8)		
Disagree	330 (27.1)	104 (20.6)	434 (25.2)		
Interviewee could help in reducing antibiotics resistance				15.65*	<0.001
Agree	543 (41.7)	182 (32.0)	725 (38.8)		
Disagree	758 (58.3)	386 (68.0)	1144 (61.2)		

* 223 interviewees did not know what drug resistance was and were excluded from analysis

3). Although 50% of each group were indifferent to doctors' antibiotic-prescribing behaviour, the local-born were more likely to prefer more cautious doctors. Likewise, although <40% of all respondents thought that they could help prevent drug resistance, the local-born were more likely to agree.

The immigrants were more likely to agree that (1) more antibiotics might weaken the body's immunity, and (2) antibiotic resistance was a serious problem in Hong Kong.

Behaviour

A frequent comment in the focus groups was that doctors seldom mentioned information about antibiotics (eg nature of the drug, reason for taking) apart from reminders to complete the full course. Trust in their doctor was the main reason for a participant's passive acceptance. A few expected to receive antibiotics on cultural or economic grounds:

> "This could be seen as a value-for-money.... And during most of the time, my family members would only take the drugs for 2 days, then illness and pain gone...." (FG6.P5_p2)

> "During the time when my children and I were still in China, my daughter got sick and she was given intravenous infusion to tackle her fever. It was really effective to control her fever. To almost every child and adult in the mainland, when they got sick, they always received this kind of treatment. However, in Hong Kong, doctors tend to prescribe ordinary drugs, not to mention antibiotics. It took longer to recover from the ailments." (in-depth interview, immigrant from Mainland China aged 36 years)

In addition to failure to complete the full course of treatment, other examples of inappropriate use of antibiotics included buying over-the-counter drugs without a prescription, and keeping left-over drugs.

> "I didn't have doubt about [the doctor and/or medicine], but I felt getting better, up to 70% and 80%, then took no more." (FG5.P3_p12)

> "Those drugs are bought [from a drug store] for prevention.... My home always has some kinds of drugs." (FG1.P4_p5)

Of all the telephone interviewees, <10% had ever requested antibiotics from their doctors, kept left-over antibiotics for future use, or bought antibiotics over the counter (Table 4). The immigrants were more likely to buy antibiotics over

TABLE 4. Responses to questions on behaviour

Question	No. (%) of participants			χ^2	P value
	Local-born	All immigrants	Total sample		
Ever asked doctor for antibiotics				0.590	0.442
Yes	136 (8.3)	76 (9.4)	212 (8.7)		
No	1493 (91.7)	735 (90.6)	2228 (91.3)		
Accepted doctor's offer of antibiotics				0.855	0.355
Yes	464 (62.5)	194 (59.3)	658 (61.6)		
No	278 (37.5)	133 (40.7)	411 (38.4)		
Kept left-over for future use				1.642	0.200
Yes	93 (6.1)	54 (7.6)	147 (6.5)		
No	1443 (93.9)	657 (92.4)	2100 (93.5)		
Treated with antibiotics during last common cold				2.805	0.094
Yes	204 (13.6)	123 (16.3)	327 (14.5)		
No	1298 (86.4)	631 (83.7)	1929 (85.5)		
Finished the full course				190.159	<0.001
Always	1071 (69.1)	462 (42.1)	1533 (57.9)		
Not always	480 (30.9)	635 (57.9)	1115 (42.1)		
Expected but did not ask				0.079	0.410
Yes	311 (21.4)	153 (22.0)	464 (21.6)		
No	1141 (78.6)	544 (78.0)	1685 (78.4)		
Bought antibiotics over the counter				5.356	0.021
Yes	112 (6.9)	78 (9.6)	190 (7.8)		
No	1518 (93.1)	732 (90.4)	2250 (92.2)		

TABLE 5. Regression models with birthplace, sex, age, and education as independent variables

Knowledge, attitude, and behaviour	Coefficient (95% CI) relative to local-born*	
	All immigrants	New immigrants
Total knowledge score	-0.467 (-0.721, -0.213)	-0.578 (-1.094, -0.061)
Can help preventing antibiotic resistance	1.307 (1.048, 1.630)	0.916 (0.590, 1.420)
Always finish the full course of antibiotics	1.207 (0.978, 1.490)	0.696 (0.465, 1.044)
Keep the left-over antibiotics	0.697 (0.481, 1.010)	2.490 (1.385, 4.477)
Buy over-the-counter antibiotics	0.601 (0.436, 0.829)	2.205 (1.230, 3.953)

* Slope coefficient for total knowledge score and odds ratios for all other dependent variables

the counter and the local-born were more likely to finish the full course. Apart from these two, there was no significant difference in the general behaviour toward antibiotics between the two groups. For young adults, there was no difference between the local-born and the immigrants in their responses to these behaviour questions.

Regression models

The knowledge, attitude, and behaviour items that differed significantly between the local-born and the immigrants were put into regression models. The independent variables included age-group, gender, education level, and immigration status. After adjusting for these variables in a linear regression, the total knowledge score was associated with birthplace (all immigrants, P<0.001) or new-immigrant status (P=0.028) [Table 5]. For attitude items, only the ability to help prevent antibiotic resistance was associated with birthplace (but not for new-immigrants) in the logistic regression. There was no association between the local-born and immigrants (all or new)

for completing a full course of antibiotics. All the immigrants were more likely to buy antibiotics over-the-counter, and new immigrants were more likely to keep the left-over drugs.

Discussion

In focus-group discussions, some participants who were recent immigrants (mostly about 5 years) had different views to others. However, the telephone survey showed that birthplace was not associated with attitude or behaviour (except for knowledge) toward antibiotics after adjusting for age and education. The only difference between the local-born and the new immigrants was in the behaviour of buying antibiotics over the counter and keeping left-over drugs, although <10% of them did so. Age and education were the main determinants of the public's knowledge, attitude, and behaviour toward antibiotics.

Hong Kong has been, and still is, an immigrant society. Its population was four million in 1970 and reached seven million in 2010. Its birth rate is among the lowest in the world: declining from 16.8 live births per 1000 population in 1981 to 7.0 in 2003. A very large proportion of the Hong Kong population comprises immigrants who are Hong Kong locals but not locally born. Among the young adults aged ≤40 years, there was no difference between the local-born and the immigrants in their knowledge, attitude, and behaviour toward antibiotics. It is probable that younger people were more receptive to new knowledge and concepts, and more ready to change their behaviour, whereas the elderly 'Hong Kong locals' retained their old ideas and habits.

In this study, 14.5% of people were prescribed antibiotics for their last URTI, very different from the 27.5% reported in 2002 from a group of selected doctors[3] and the 23.7% reported in 2008.[2] This might suggest a decreasing antibiotic prescription rate. In the 2008 report, 78% of the interviewees completed the full course of antibiotics, but in this study only 57% *always* did so. About 70% of the interviewees in 2008 were deemed to have adequate knowledge (3 out of 5), but in this study the mean total score on knowledge was 7.8 out of 16 indicating inadequate knowledge about antibiotics. Nonetheless, 9% of the interviewees in 2008 and 7.8% in this study bought antibiotics without a prescription.

Although >80% of the interviewees agreed that cautious use of antibiotics could help prevent drug resistance, <40% agreed that they could help. This was probably because in most consultations, antibiotics were simply prescribed without further explanation rather than being actively requested. Less than 10% of all the interviewees had ever asked their doctors for antibiotics, kept left-over antibiotics for future use, or bought antibiotics over the counter. Nonetheless, <60% always finished the full course, which is unacceptable.

In the telephone survey, the characteristics of the non-respondents could not be obtained. It was uncertain whether or how the non-response rate would affect the results although the response rate of 68.3% was satisfactory. It is possible that the most recent new-immigrants who did not speak Cantonese were excluded from this study.

Conclusion

The main determinants of the general public's knowledge, attitude, and behaviour toward antibiotics were age and education. New immigrants performed comparably with the local-born. The Hong Kong public's knowledge about antibiotics was inadequate. The awareness of their role in preventing AMR should be raised.

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