2nd MOLECULAR AND DIAGNOSTIC MICROBIOLOGY CONGRESS

CONGRESS PROCEEDINGS AND PROGRAMME

April 21 - 25, 2002
International Meeting

Merit Limra Hotel
Kemer, Antalya -TURKEY

Ankara Microbiology Society
&
Hacettepe University Faculty of Medicine
Department of Microbiology and Clinical Microbiology

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GENERAL INFORMATION

Registration and Info Desk:
Congress Registration Desk for the registration and info services will be at your service on Sunday, April 21 at 09:00. Registration fee includes; participation to the meetings, congress bag, congress proceedings, 4 night accomodation, breakfast and dinner. Congress secretariat can not guarantee to provide congress bag and abstract book to the on-site registered partiticipations.

Badges:
All the participants and accompanying persons will have badges. The badges will be in different colors for participants, accompanying persons and industrial participants. The guests without badges will not be able to attend the congress social programme.

Language:
The official language of the congress is English. There will be simultaneous translation to Turkish.

Attendance Certificate:
Attendance certificates will be distributed on April 24, 2002.The participants who will leave early are kindly requested to inform the info desk or must authorize somebody for receiving the certificate.

Slide Center:
The speakers should deliver their slides or other presentation materials to the persons in charge at least 2 hours prior to the session time, for morning presentations speakers should deliver their slides to the persons in charge a night before. Slides should be placed in plastic frames.

Congress Official Hotel:
Accomodation is going to be done on half board basis from April 21, 2002 to April 25, 2002 including 4 nights. The participants should inform the info desk about their requests. All the services other than the package programme services; extra drinks and mini bar will be charged to the participants. The organization agency will not have any responsibility on that subject.

Posters:
First categorie posters will be presented on April 22, 2002, second categorie posters on April 23, 2002 and third categorie posters on April 24, 2002 at 09:00 in poster room, and they should be removed at 17:30. Poster presenters should be at the exhibition hall, by their posters' site at the time intervals noted below:

April 22, 2002 : 16:00-17:00
April 23, 2002 : 12:30-13:30
April 24, 2002 : 12:30-13:30

2nd MOLECULAR AND DIAGNOSTIC MICROBIOLOGY CONGRESS PRECONGRESS WORKSHOP PROGRAMME

Saturday, April 20:

9:30-10:00: Introduction, Molecular laboratory methods used in research and diagnosis of infectious diseases.
Tanıl Kocagöz

10:00-11:30: DNA isolation and purification of *M. tuberculosis*, from sputum.
Rıza Durmaz, Selami Günal

11:30-12:00: Restriction enzyme analysis.
Rıza Durmaz, Selami Günal

12:00-13:00: Break.

13:00-14:30: PCR- Classical Methods; Fluorometric Amplification, Mutation Detection.
Tanıl Kocagöz, Hülya Aydoğan

14:30-14:45: Break

14:45-17:00: Polyacrylamide gel electrophoresis and protein transfer to nitrocellulose membrane for Westernblot.
Fatih Köksal, Fügen Yarkın

17:00-17:15: Break

17:15-18:00: Evaluation of the results of restriction enzyme analysis and overnight electrophoresis.
Rıza Durmaz, Selami Günal

Sunday, April 21:

9:00-10:45: Western-blot: Staining of nitrocellulose membranes.
Fatih Köksal, Fügen Yarkın

10:45-11:00: Break

11:00-12:00: Southernblotting. Transfer of DNA from agarose gel to nitrocellulose membrane.
Rıza Durmaz.

12:00-13:00: Break

13:00-15:00: Agarose gel electrophoresis and isolation of plasmide.
Tanıl Kocagöz, Hülya Aydoğan

CONGRESS PROGRAMME

Sunday, April 21

17:50-18:00
Opening
Ayfer Gunalp, M.D.
Hacettepe University, Medical School,
Dept. Of Microbiology and Clinical Microbiology

18:00-18:50
Opening Session

Session Chairman: Ayfer Günalp, M.D.

"Quorum sensing and genetic transformation in Streptococci"
Donald Morrison, Professor of Biological Sciences
Laboratory for Molecular Biology
University of Illinois at Chicago, Chicago

CONGRESS PROGRAMME

Monday, April 22
9:00-9:50

Session Chairman: Burçin Şener, M.D.

"Evolution and global epidemiology of multidrug resistant clones of *Streptococcus pneumoniae* and *Staphylococcus aureus."*
Alexander Tomasz, Ph.D.
The Rockefeller University

9:50-10:40

Session Chairman: Rıza Durmaz, Ph. D.

"Methods used for the molecular epidemiology of tuberculosis."
M. Donald Cave, Ph.D.
University of Arkansas for Medical Sciences

10:40-11:10 Coffee Break

11: 10-12:00

Session Chairman: Gülşen Hasçelik, M. D.

"Wide-spread strains of *Mycobacterium tuberculosis* discovered by molecular epidemiology."
M. Donald Cave, Ph.D.
University of Arkansas for Medical Sciences

12:00-13:30 Lunch and Diomed Satellite Symposium

13:40-14:50

Session Chairman: Selim Badur, Ph. D.
"Rapid identification of *Mycobacteria* to the species level by PCR and restriction enzyme analysis."
Kaya Köksalan, M.D.
İstanbul University, Medical School

"Public sequence database for differentiation of Mycobacterium species."
Dag Harmsen, Ph.D.
Head R&D, CREATOGEN Diagnostics

"Rapid determination of rifampin resistance in Mycobacterium tuberculosis *by PCR and fluorescense resonance energy transfer."*
Tanıl Kocagoz, M.D, Ph.D.
Diomed , Head R&D

14:50-15:10 Coffee Break

15:10-16:00

Session Chairman: Yakut Akyön, Ph. D.

"Genome evolotion and the emergence of metronidazole resistance in *Helicobacter pylori."*
Douglas E. Berg, Ph.D.
Washington University Medical School

CONGRESS PROGRAMME

Tuesday, April 23

9:30-10:20

Session Chairman: Okan Töre, M. D.

"Proteases of malaria parasites: New targets for chemotherapy."

Phil Rosenthal, M.D.
University of California, San Francisco

10:20-10:40 Coffee Break

10:40-11:40

Session Chairman: Sibel Ergüven, Ph. D.

"Developments in laboratory diagnosis of amebiasis."
Mehmet Tanyüksel, M.D.
G.A. T .A. Department of Parasitology, Ankara

"Malaria Vaccines."
Ülgen Zeki Ok, M.D., Ph. D.
Celal Bayar University, Department of Parasitology

12:00-13:30 Lunch

13:40-14:30

Session Chairman: Tevfik Cengiz, M.D.

"Molecular markers of Antimalarial drug resistance in Uganda."
Phil Rosenthal, M.D.
University of California, San Francisco

14:30-14:50 Coffee Break

14:30-15:20

Session Chairman: Fügen Yarkın, M.D.

"Isothermal DNA amplification-ramification amplification"
David Y. Zhang, MD, Ph.D.
Director, Molecular Pathology Laboratory
Mount Sinai School of Medicine

CONGRESS PROGRAMME

Wednesday, April 24

9:00-9:50

Session Chairman: Ferda Tunçkanat, M.D.

"Currently avaliable and novel antifungal drugs: Modes of action and mechanisms of resistance."

Sevtap Arıkan, M.D.
Hacettepe University, Medical School,
Dept. Of Microbiology and Clinical Microbiology

9:50-10:40

Session Chairman: Mehmet Ziya Doymaz, M.D.

"Vaccines against Herpes viruses."
Barry T. Rouse, Ph.D.
University of Tennessee

10:40-11 : 10 Coffee Break

11: 10-12:00

Session Chairman: Fatih Köksal, M.D., Ph. D.

"Applications of microarray technology to bacterial expression patterns."
Donald Morisson, Ph.D.
University of Illinois, Chicago

12:00-13:30 Lunch and Satellite Symposium

Free Time

Thursday, April 25

9:00-9:50
Session Chairman: Şemsettin Ustaçelebi, Ph. D.

"Viruses and immunopathology."
Barry T. Rouse, Ph.D.
University of Tennessee

9:50-10 : 10 Coffee Break

10:10-11:00
Session Chairman: Dürdal Us, Ph.D.

"Molecular biology of hepatitis B virus and its diagnosis by conventional tests."
Şemsettin Ustaçelebi, Ph. D.
Hacettepe University, Medical School,
Dept. Of Microbiology and Clinical Microbiology

"Molecular diagnosis of hepatitis viruses."
Selim Badur, Ph. D.
İstanbul University, Medical School,
Dept. Of Microbiology and Clinical Microbiology

11: 10-12:00 Closing Session; Presentation of Poster Awards

CONFERENCES

RAPID IDENTIFICATION OF MYCOBACTERIA TO THE SPECIES LEVEL BY POLYMERASE CHAIN REACTION AND RESTRICTION ENZYME ANALYSIS

Köksalan OK
University of İstanbul , İstanbul Medical Faculty, İstanbul, TURKEY

The resurgence of tuberculosis and the growing number of multidrug-resistant *Mycobacterium tuberculosis* strains has reinforced the need for development of new diagnostic tools with shorter detection and identification times.Conventional biochemical and phenotypic tests for mycobacterial identification are laborious and time-consuming and require specialized testing that is beyond the capability of most clinical laboratories. High-performance liquid chromatography (HPLC) or thin-layer chromatography are limited by their high initial investments and by the need for standardized growth conditions. Genotypic mycobacterial identification tools are of ever-increasing importance due to their rapidity and unequivocal results. Nucleic acid hybridization method (Accuprobe, Gen-Probe, Inc., USA) requires several probes and can identify only a limited range of mycobacterial species. Though a powerful technique of differentiating species, 16S rRNA sequencing is labor-intensive and sophisticated, and therefore, difficult to implement for routine use in many clinical laboratories.

A rapid, user-friendly and unambigious identification method, using polymerase chain reaction and restriction enzyme analysis (PRA) was described by Telenti et al. This method is based on the evaluation of the gene encoding the 65 kDa heat shock protein(hsp65) which is possessed by all mycobacteria. Cleaving the amplified DNA into fragments, using restriction endonucleases BstEII and HaeIII and then separating the fragments on an agarose gel do species identification of mycobacteria. The identification of the organism in question is obtained by comparing the resulting restriction enzyme pattern with that of known mycobacterial reference strains.

After a two-years experience some shortcomings of the method has been detected;

a. Not all of the mycobacterial species were included in the algorithms of the previous studies
b. The apparent fragment sizes given in the previous studies differ greatly from the true fragment sizes (sometimes >10bp)
c. Restriction Enzyme Analysis based on two endonucleases is not always sufficient to differentiate two isolates of different species from each other (e.g. M. gastri GenBank accession no U 17931 gives the same restriction pattern with M. kansasii U 17946)
d. Currently available molecular size markers do not have bands the same size as with the mycobacterial strains which leads to difficulties interpreting the results and necessitates computer-guided calculations.

A multi-centered study was carried out to overcome the shortcomings mentioned above and the results will be discussed in this session.

RAPID DETERMINATION OF RIFAMPIN RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* BY PCR AND FLUORESCENCE RESONANCE ENERGY TRANSFER

Kocagöz T[1], Sarıbaş Z[2]
[1]Diomed A.Ş. Department of R&D and Acıbadem Health Group, İstanbul
[2] Hacettepe University, Medical School, Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

Summary

Fluorescence Resonance Energy Transfer (FRET) is the transfer of energy between two fluorescent molecules, closely located. Two DNA probes one labeled with a fluorescent dye at 3' end and the other at 5' end that bind very close to each other on an amplified DNA molecule during PCR, enable the fluorescent detection of amplification and presence of mutations. The change in melting temperature of these probes due to mismatches are critical for the detection of mutations by FRET. We have applied this technique to identify mutations in *rpoB*, encoding the B subunit of RNA polymerase, that lead to rifamycin resistance. It was possible to determine rifamycin resistance in 92.7% by PCR and FRET in *M. tuberculosis* isolates determined as resistant to rifamycins by culture.

FRET: The Technique That Enabled PCR Directly to Quantitate DNA and Detect Mutations

By enabling in vitro amplification of DNA, Polymerase Chain Reaction (PCR) provided the means for easy detection of many infectious agents that are otherwise impossible or very hard to detect by classical methods. It also became a source for DNA needed for many molecular methods like the ones used in mutation analysis for identification of human genetic diseases and drug resistance in infectious agents (1-4). However it was not possible to quantify directly the amount of DNA or RNA in a sample, which is crucial for measuring gene expression or following the response to treatment in chronic infections or identify mutations until fluorometric detection during PCR became available.

The amplification of DNA can be followed in real time by fluorescence produced from two probes that bind closely on one of the DNA strands produced during PCR amplification (5). For this purpose two DNA probes one labeled with a fluorescent dye (fluoresceine) at 3' end and the other (LC-Red640 or LC-Red705) at 5' end complementary to close regions of amplified DNA is prepared. When PCR product increases during amplification more and more probes start to bind close to each other on these DNA strands. The reaction tubes are enlightened by blue light which is absorbed by fluorescein and emited as green fluorescent light. Red640 or Red705 which are not activated by blue light, are activated by this green light and emit red light at a wavelength of 640 and 705nm respectively. The produced red light is followed as an indicator of amplification (6) (Fig 1).

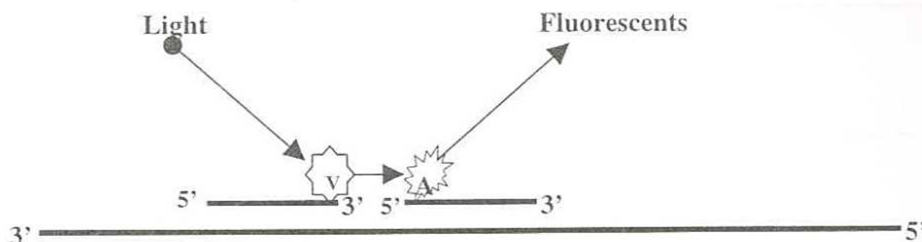

Fig 1: Fluorescence production of labelled probes which are bound to target DNA by FRET (v: anchor, a:detector).

Mutation Detection by FRET Probes

The 3' fluorescein labeled probe is called the "anchor probe" and is designed to bind stronger to its complementary strand compared to the 5' labeled probe which is called the detector probe. To identify mutations detector probe is designed to cover the region where mutations are expected. At the end of amplification, maximum amount of probe bind close to each other and maximum amount of fluorescence is produced. Then the tubes are heated slowly and the fluorescence is followed. At a certain temperature the detector probe falls off the complementary strand and the fluorescence drops sharply. A melting curve is obtained by following the fluorescence. The temperature at which maximum drop in fluorescence occurs is called the melting peak. The melting peak of each probe binding in a completely complementary manner is a certain specific temperature. If there is a mutation on probe binding site, then a mismatch occurs loosening the binding and creating a decrease in melting peak temperature (5, 6).

rpoB Mutations Leading to Resistance to Rifamycins in *M. tuberculosis*

Rifampin and other rifamycin derivatives rifabutin, rifapentine, rifalazil inhibit RNA polymerase by binding to beta subunit. Mutations at *rpoB* creating amino acid changes especially at positions 513, 516, 526, 531 and at amino acids close to these regions lead to resistance to rifamycin derivatives (7-11). However all changes do not create resistance equally to all rifamycin derivatives. For example while changes at amino acid 531 create almost always resistance to all rifamycin derivatives, changes at amino acid 516 create resistance to rifampin and rifapentine, while the bacteria stay susceptible to rifabutin and rifalazil (12). For this reason it may be important to differentiate the type of mutation as well as detecting it in *M. tuberculosis* isolates.

Strategy for Detecting and Differentiating the rpoB Mutations by PCR and FRET

We have designed anchor and detection FRET probe pairs for identification of mutations in *rpoB* of *M. tuberculosis*. One detection probe which was labeled with LC-Red 640 covered the 526, 531 region and the other which was labeled by LC-Red 705 covered the 513, 516 region (Fig 2). Using these probes and LightCycler (Roche Diagnostics) we have followed the amplification of a 305bp region of *rpoB* using primers TbRif1: 5' CAG ACG TTG ATC AAC ATC CG 3' and TbRif2: 5'TAC GGC GTT TCG ATG AAC 3'. At the end of amplification we have obtained melting curves by heating the reaction tubes slowly from 60 to 95°C and following the fluorescence

at 640 and 705nm wavelength. A drop of more than 2°C in melting peak was considered as an indication of a mutation (Fig 3).

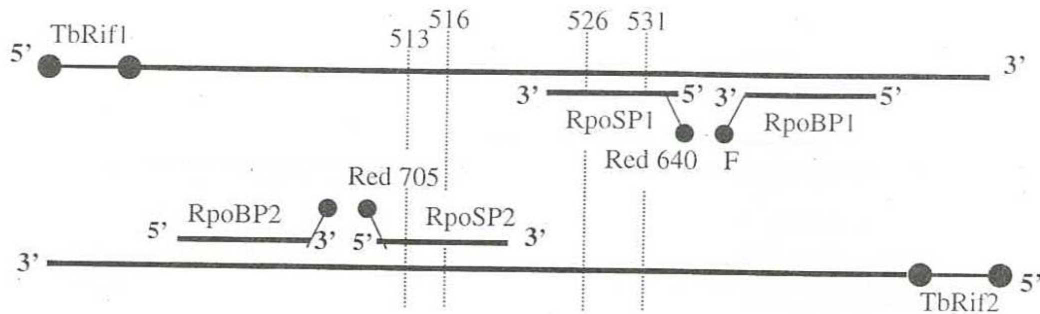

Fig 2: The 305 bp part of rpoB which is amplified by TbRif1 and TbRif2 primers and annealing sites of the anchor and detector probes, 513, 516, 526 and 531 codon positions which are the most frequent mutation sites.

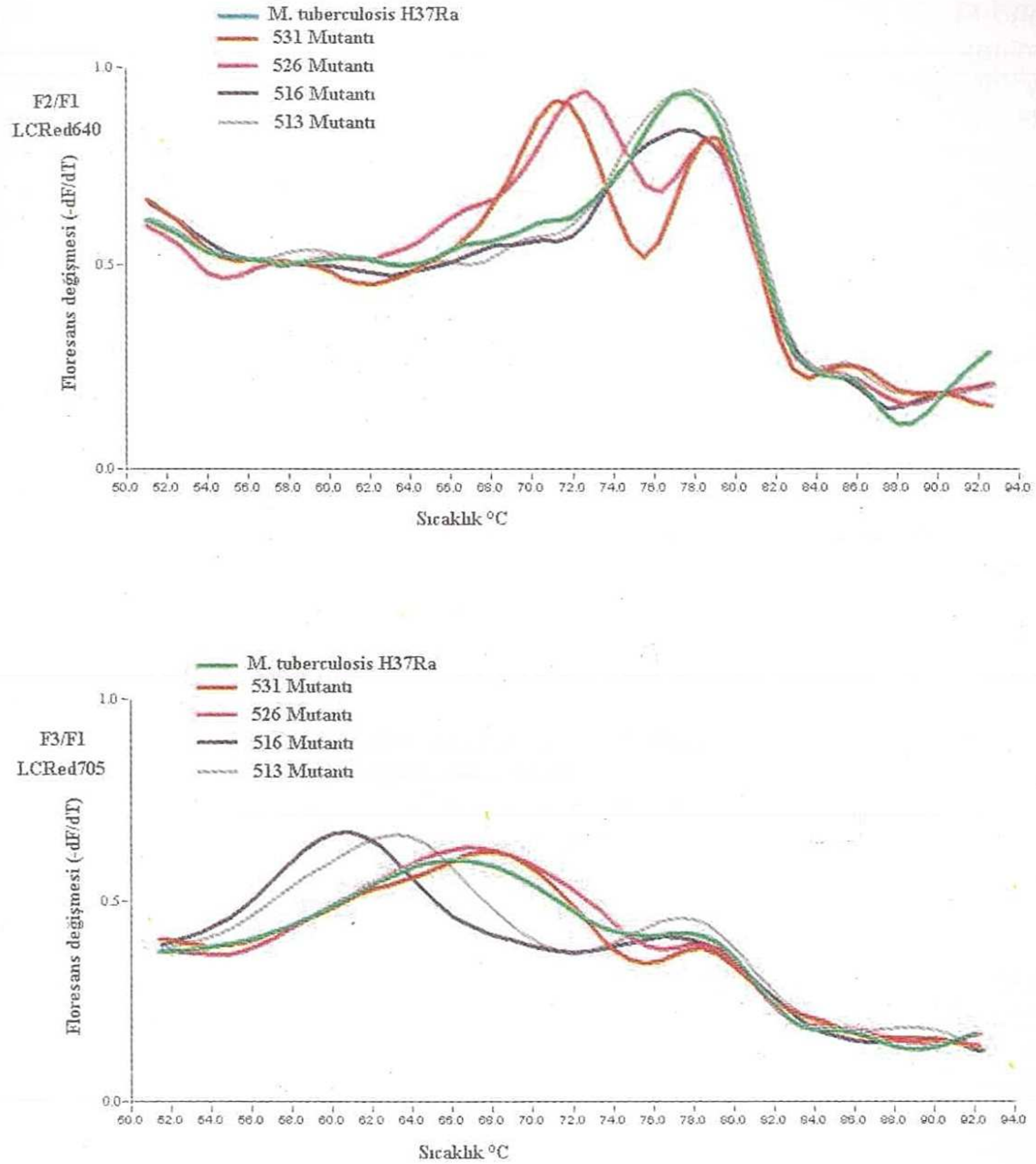

Fig 3: Melting curves of rifampicin sensitive and resistant *M. tuberculosis* H37Ra mutant strains.

Using PCR-FRET it was possible to determine rifampin resistance in 92.7% of 96 isolates previously determined as rifampine resistant by culturing in drug containing media. An other important result obtained was that 46.9% of rifampin resistant strains were susceptible to rifabutine.

Conclusion

PCR and FRET provides the opportunity to detect rapid and easy determination of rpoB mutations leading to resistance to rifamycin derivatives. It is also capable to identify the type of mutation which may be very useful in choosing the rifamycin derivative that should be used for treatment since different mutations cause different level of resistance for each rifamycin derivative. For example, according to the results obtained in this study, rifabutine may be an important alternative in the treatment of patients infected with rifampin resistant strains. PCR-FRET technology may easily be adapted to determine resistance to other antituberculosis drugs in *M. tuberculosis* isolates (13).

References

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GENOME EVOLUTION AND THE EMERGENCE OF METRONIDAZOLE RESISTANCE IN *HELICOBACTER PYLORI*

Berg DE[1], Boren T[2], Dailide G[1], Dailidiene D[1], Gilman RH[3], Hoffman PS[4] Jeong JY[1], Kersulyte D[1], Mukhopadhyay AK[1]

[1]Department of Molecular Microbiology and Department of Genetics, Washington University Medical School, St. Louis, MO, USA

[2]Department of Oral Biology, Umea University, Umea, SWEDEN

[3]Department of Pathology, Universidad Peruana Cayetano Heredia, Lima, PERU

[4]Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, CANADA

Summary and Overview

Helicobacter pylori (Hp) chronically infects the gastric mucosa of billions of people worldwide, causes peptic ulcer disease, increases the risk of gastric cancer, and contributes to vulnerability to several other disease conditions. Hp is one of the most genetically diverse of bacterial species, and different genotypes predominate in different human populations. These findings have led to new models of the origins and evolution of Hp as a human pathogen, and may have important human health consequences. Resistance to anti-Hp drugs, especially metronidazole (Mtz), is a major problem clinically. Recent experiments showed that Mtz resistance results from mutational inactivation of chromosomal genes encoding one, and in some cases, two nitroreductases (depending on background genotype), not from new plasmid- or transposon-borne resistance determinants. These nitroreductases convert Mtz from harmless prodrug to bactericidal agents which are also mutagenic, and that thus may speed bacterial adaptation and the emergence of resistance to other antibiotics.

Introduction

Helicobacter pylori is a gram negative microaerophilic bacterium that chronically infects the gastric mucosa of more than half of all people worldwide. It is a major cause of gastritis, of duodenal ulcers and of gastric ulcers, and an early risk factor for gastric cancer, even though many infections are asymptomatic (for reviews, 1-3). Other clinical conditions that have also been linked to *H. pylori* infection include susceptibility to diarrheal disease, malnutritition, iron deficiency anemia, and recurrent abdominal pain in children (4-7).

Infection by Hp occurs preferentially in early childhood and tends to last for years or decades despite inflammatory responses and other host defenses. Infection is most common in developing countries, and is related to poor water quality, overcrowding, and other as yet undefined socio demographic and environmental factors. Hp is specific to surfaces of gastric mucosal cells and the overlaying mucin, an ecological niche in which few other bacterial species can survive. It does not grow on other mucosal tissues such as intestinal mucosae, and may not proliferate or even persist long in the environment. In accord with this, epidemiologic studies have indicated that Hp is transmitted preferentially within families, probably often by food, water, fingers, etc., which has been recently contaminated with fecal and/or vomitus material (8-9). Features of Hp likely to be important in the establishment and maintenance of infection include adherence to epithelial cell surfaces, bacterial motility and chemotaxis, tolerance of mildly acidic conditions, avoidance of clearance by immune and inflammatory host defenses, and a potent urease. The full genomic sequences of two H. pylori strains from ethnic European patients are now available (10-11).

Because Hp resides primarily on surfaces of gastric epithelial cells and in the overlying mucin layer, which is not highly permeable to solutes from the gastric lumen, it is attractive to imgaine that it feeds primarily on exudates from tissue that has been damaged by toxins that Hp produces or by inflammatory responses that it elicits (12). In this view, Hp may need to cause enough tissue damage to be well nourished, but ideally not so much that it provokes clearance by host responses to infection or loss of its special gastric epithelial niche during years of chronic infection. This challenge would be increased by genetic and physiologic diversity among us humans in our host responses to proinflammatory stimuli that Hp infection generates. The patchiness of Hp growth and localized nature of the host inflammatory response (13) may also contribute to Hp's long term success: any mutant or recombinant subclone that triggered too strong a response would be killed preferentially, or be lost because of severe damage to the patch of gastric epithelial-type tissue with which had associated.
Here we review current understanding of the population genetic structure and evolution of H. pylori, and the mechanisms by which it becomes resistant to the clinically useful antibiotic metronidazole (Mtz).

1. Hp genome diversity and population genetic structure :
H. pylori is one of the most diverse of bacterial species. Any two independent isolates are usually easily distinguished by simple arbitrarily primed PCR (RAPD) fingerprinting or DNA restriction analysis (14), and independent clinical isolates typically differ from one another by some 3-5% on average in sequences of essential housekeeping genes (15). Even more diversity is seen in genes for adhesins, toxins or other proteins that may interact directly with host tissues; this might reflect selection for different motifs of these proteins in different human societies.
Several determinants have been identified as virulence-associated epidemiologically, and are often found together in clinical isolates (Fig. 1).

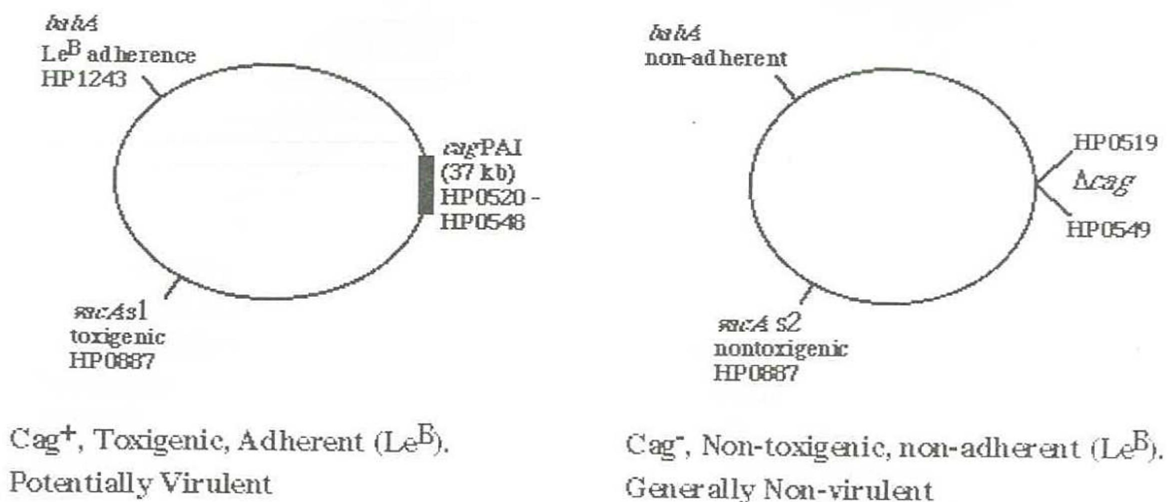


Fig. 1. *Diagram of genomes of the two major types of Hp strains encountered in North America, Europe and parts of Asia. These two types are defined from the perspective of genes likely to contribute to disease. Recombinant types, containing just one or two of the determinants diagrammed on the left are also seen, but their frequencies are far less than would be expected by chance alone.*

Of particular note are the ~40 kb cag pathogenicity island (PAI), the vacA (vacuolating cytotoxin) gene, and the BabA (LeB-specific) adhesin (Fig. 1). Only about one-half to two-thirds of European or North American strains carry the cag PAI, but nearly all of these strains are associated with overt disease. Other strains generally lack the cag PAI entirely, and are generally isolated from persons with more benign infections (1, 16). Many cag PAI genes encode components of a type IV secretion system, which allows stimulation of synthesis of cytokine IL-8 in the gastric epithelial cells to which the Hp are bound. IL-8, in turn, recruits and activates neutrophils, and thereby helps provoke severe and potentially injurious inflammatory responses that may contribute importantly to overt disease (1, 16-18).

A large (120-140 kD) protein, designated CagA, is encoded within the cag PAI, is immunodominant, but is not needed for induction of IL-8 synthesis. It is translocated to target epithelial cells by the cag type IV secretion system, however, and is tyrosine-phorphorylated there, where it seems to cause changes in cytoskeletal structure and in cell developmental programs, perhaps by affecting the phosphorylation of cellular signalling proteins (19,20). The immunodominance of CagA protein and its association with virulence has made it attractive for serologic surveys to detect potentially virulent vs. benign infections, although we would advise special caution in interpretation of seroprevalence data. For example, one recent survey concluded that cagA+ Hp strains are disappearing more rapidly than cagA- strains from the general population (20), and another concluded that they are cleared more effectively by antimicrobial therapy (21). A need for caution in making these conclusions comes from findins that people can be chronically infected by two unrelated strains, and that one can be cag+ and the other cag- (22), and hte likelhood that the risk of mixed infection probably reflects the overall Hp infection risk. Because serologic tests would score such infections only as cag+, these outcomes might simply reflect a disproportionate decrease in frequency of mixed infections as the overall risk of infection declines or eradication of all but one of several coinfecting strains during partially effective therapy. Each of these circumstances would allow correct serologic diagnosis of more of the infections that involve cag- strains. We

note that mixed infections can, in contrast, be detected reliably by PCR (with primers specific for the cag "empty site" and other primers specific for cag PAI genes) if biopsy or gastric juice or potentially other patient samples are available (see 16,22).
Most cag+ strains contain toxigenic "s1"-type (signal sequence) alleles of vacA, a gene whose protein product forms pores in plasma membranes, alters endo-lysosomal function, induces apoptosis and epithelial monolayer permeabilisation, interferes with antigen presentation, etc (1, 23). Most Cag- strains contain "s2" type alleles, whose encoded protein products are non-toxigenic. This non-toxigenicity has been traced to features of the amino termini of the mature VacA protein, and this allows the s1 and s2 types to be distinguished easily by length of products of s region-specific PCR. VacA toxins also differ in target cell specificity, a property controlled by motifs in the middle region of VacA protein (24). It is attractive to imagine that cell type specificity may also affect the specificity or severity of colonization or disease.
The several types of adhesins of Hp constitute a third determinant that are likely to contribute variously in colonization, persistence or disease (25-29) . The most well studied adhesin is BabA, which was first isolated from a strain from an Amerindian (Native) Peruvian. It is specific for the LewisB (terminal branched fucose) antigen, a carbohydrate structure found in abundance in the gastric epithelium (25). Only a subset of Hp strains (predominantly those that are cag+ and vacAs1) exhibit BabA adhesin activity, and these "triple positive" genotypes seem to be most strongly associated with severe pathology and overt disease(see 28-30). New carbohydrate structures, including sialylation, appear during Hp-induced inflammatory responses. Some of Hp's other adhesins may help it cope with these host defenses (31 T. Boren, pers commun).
It is noteworthy, that cag+ status, the capacities for BabA adherence and vacuolation, are determined by loci that are far from each other in the chromosome, yet these genes are linked epidemiologically (Fig. 1). Mutational tests, however, have shown that the cag PAI is not needed for adherence or for toxigenicity (16,25). Thus, these three determinants (cag, toxigenic vacA and BabA) may reflect co-adapted gene complexes. Strains carrying all three determinants, and also strains lacking all three determinants, may each be favored over intermediate strains that carry just one or two of them. Current ideas of how each of these determinants beenfits Hp do not predict how the many apparently non-virulent strains of Hp to establish their own persistent but benign gastric mucosal infections.

Origins of Hp diversity : As noted above, Hp is extremely diverse, even at the level of single nucleotide polymorphisms (SNPs) in conserved essential genes. Much of this extraordinary diversity has been ascribed to frequent mutation (32,33) and to DNA transfer and recombination between divergent strains (15,22,34). We suggest that three other factors also contribute to Hp's great diversity: (i) its preferentially intrafamilial mode of transmission; (ii) the many years during which a strain must cope with its particular host's inflammatory and other responses to infection; and (iii) differences among humans in traits that could be important to individual strains. Because of these features less fit mutant and recombinant genotypes may accumulate if they happen to be ingested by a host not already carrying Hp of a more fit genotype. Mutant or recombinant derivatives with compensatory changes that improve the strain's fitness in this particular host will often arise and be selected. As in classical island populations, the stochastic nature of mutational and recombinational changes, and the many ways in which certain quantitative traits can

be modified, along with differences among hosts will tend to promote different adaptive or evolutionary trajectories in each infected person, and thereby continuous divergence from an ancestral form. Hp's highly fragmented population structure precludes effective selection for any one or few Hp genotypes that might be most fit for all humans.

Superimposed on the diversity in local population, are geographic differences in predominant genotypes. This is illustrated by our DNA sequencing and phylogenetic analysis of single nucleotide polymorphisms (SNPs) in the mid region of the vacuolating cytotoxin (vacA) gene (Fig. 2; ref. 35), or by PCR analyses of a set of insertion - deletion polymorphisms at the right end of the cag PAI (Fig. 3; ref. 36).

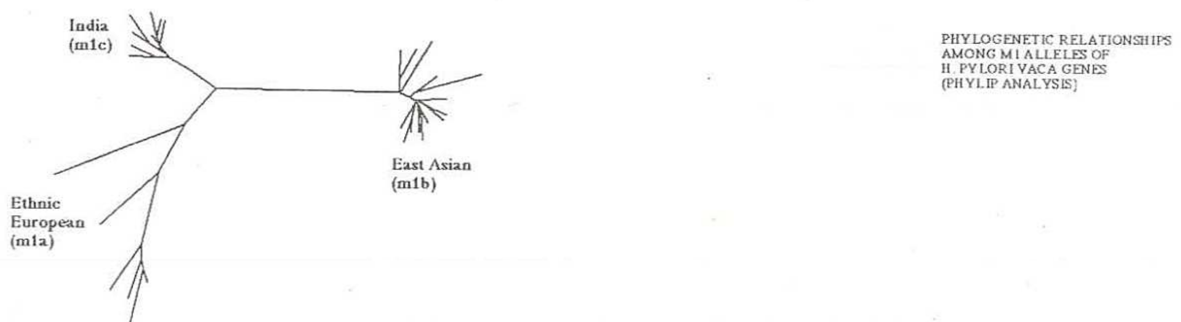
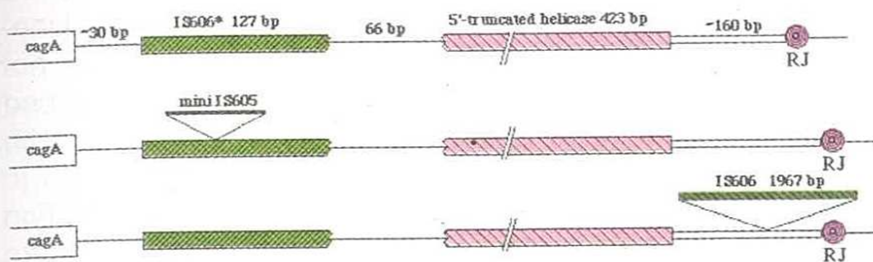


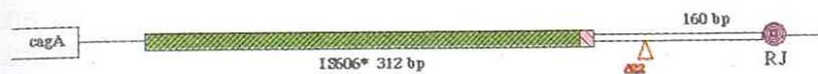
Fig. 2: *Phylogenetic tree, diagramming markedly different lineages of alleles of the m1-type middle region alleles of the vacA (vacuolating cytotoxin gene) in strains from different human societies. This mid region of vacA is of particular interest because it seems to affect the cell-type specificity of vacA action (adapted from ref. 35).*

"TYPE I"



SPAIN 33/36
PERU NATIVE 62/68
AFRICA 40/40

"TYPE II"



CHINA JAPAN 181/191

"TYPE III"

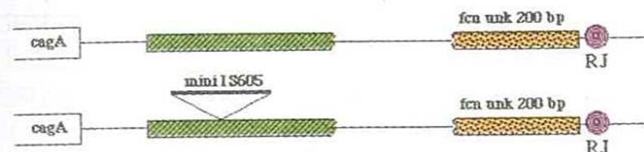


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Fig 3: *Structures of insertion-deletion motifs near the right end of the cag pathogenicity island. The major differences in sequence associated with motifs shown here, and equivalent motifs at other highly polymorphic loci are valuable in quick PCR-based screens to identify likely geographic origins of strains from different human societies (adapted from ref. 36).*

These studies indicated that the strains predominating in East Asia may be distinct genetically from those predominating in Spain, and that those predominating in India represent yet a third phylogenetic group. It was striking that most strains from Native Peruvians were closely matched to those of Spain, not Asia, since the these Amerindians descend primarily from hunter-gatherers who migrated from Asia to The Americas some 30,000 years ago. Similar geographic clustering is seen in sequences of oipA and babA (37,38). The proteins encoded by these genes probably also interact with host tissue, and thus, conceivably, might also have been subject to selection for different motifs in different human populations. However, we have recently found that East Asian and European strains can also be distinguished based on sequences of several essential housekeeping genes (G. Dailidiene, AK Mukhopadhyay and DE Berg and associates, in prep). Thus, the geograhic differences among Hp strains are likely to be genome wide. Further study should show more clearly which geographic differences reflect selection for different phenotypes in different human populations, and thereby more clearly identify genes whose protein products may be important in the specificity of host interaction and disease. There are very striking geographic differences in patterns of Hp-associated gastroduodenal disease (e.g., higher frequencies of gastric than of duodenal ulcer in much of East Asia, vs., many fold higher frequencies of duodenal than of gastric ulcer in India). Although typically this has been ascribed to regional differences among the people or their circumstances (39), it may now be appropriate to consider that regional differences in predominant Hp genotypes might also contribute to the patterns observed.

The similarities of European and Latin American genotypes suggest that most strains of Latin America are of European origin, and derive from strains that had infected the European conquerors (the "Conquistadores") some 500 years ago (36). This thinking, in turn, invites speculation that the Asian ancestors of modern Amerindians may not have been infected with Hp. In this context, we suggest that Hp may have jumped from ancient animal hosts to humans quite recently in evolutionary time, perhaps with the start of agriculture, and that perhaps it jumped from different animal hosts in different early agricultural societies (36). An alternative view holds that Hp infection has been near-universal in humans and perhaps in our non-human primate ancestors for millions of years (40). To reconcile the present Latin American Hp genotype data with the ancient origins model of ref. 40 one might suppose that European strains brought to the New World ~500 years ago were more robust than the putative indigenous Asian-like strains, and substantially displaced them without much recombination between the new and older Asian-like strains.
In the context of this meeting, the genotypes of Hp strains of Turkey will be interesting to study. Interpretation of the data in the context of Turkey's central location in the vast Eurasian landmass, and the special history of her peoples could provide important new insights into the origins and evolution of Hp as a gastric pathogen.

2. Metronidazole (Mtz) resistance :
We have studied how Hp becomes resistant to the important anti-Hp drug metronidazole (Mtz) and in parallel the mechanisms that underly Mtz susceptibility in many strains. These studies also contribute to our understanding of how Hp interacts with adapts to its host environment.
Many Hp clinical isolates (up to 90% in some societies) are MtzR, and this resistance markedly diminishes the effectiveness of popular and affordable Mtz-based anti-Hp therapies (35, 41,42). Of historical note, Mtz was developed as an anti-parasitic drug, but was soon found to also be effective against many anaerobic bacterial pathogens (43). In many developing countries, especially, it is now often used in empirical self-medication against common, minor and mostly self-limiting diarrheal diseases and other maladies. These uses of Mtz have been sufficient to cause mutation (detailed below) and selection for Mtz resistant derivatives of resident strains.
Basis of Mtz susceptibility and resistance in *H. pylori* : Mtz and related nitroimidazoles are pro-drugs whose anti-microbial activity depends on their activation by low redox active systems to form biologically active nitroso derivatives and hydroxylamines (Fig. 4).

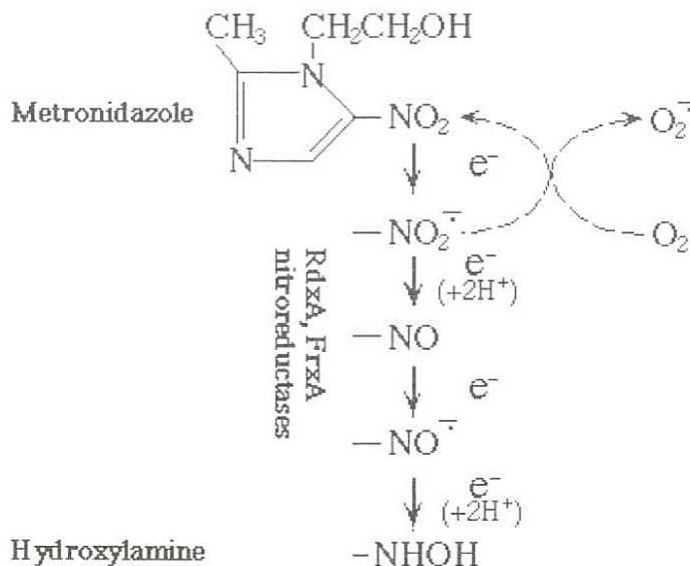

Fig. 4: *Mechanism of Mtz susceptibility and resistance in Hp. The nitroreductase enzymes RdxA and FrxA carry out a series of electron transfers that generate hydroxylamine, which is bactericidal and also mutagenic. Mtz resistance generally results from loss of function mutations at least in rdxA. FrxA is also often mutant, as detailed in the text.*

Models to explain the basis of Mtz resistance in Hp that have been considered over the years include: futile cycling of electrons between Mtz and its reduced forms, generating toxic oxygen (rather than nitro) radicals; facilitated efflux, and changes in recA protein-mediated DNA repair capacity (43), but none of these models seemed satisfactory once critically tested. We used molecular genetic approaches to understand the mechanism of Mtz resistance. First a recombinant DNA library was constructed in an *E. coli*-Hp cosmid shuttle vector, and screened to identify cosmids that rendered a Mtz susceptible Hp lab strain resistant to Mtz by DNA transformation (44). Subcloning, transformation and sequencing of DNAs from one such cosmid identified an rdxA gene containing a nonsense (null) mutant allele. The model that this suggested (Fig. 4 ; ref. 44) was further developed in a series of additional tests, as follows.

a. Sequence changes in rdxA in newly generated MtzR mutants : MtzR derivatives of a standard lab strain (26695) selected in culture generally contained mutations in or just upstream of rdxA (45). So, also, did most MtzR derivatives of the rodent-adapted SS1 strain, selected during subtherapeutic treatment of SS1-infected mice with Mtz (46). This strengthened the view that rdxA inactivation was needed to achieve MtzR, at least in these two laboratory strains.

b. rdxA mutations in MtzR clinical isolates : rdxA was amplified from 60 MtzR clinical isolates from several parts of Europe, Asia and the Americas. In each case, the rdxA DNA could transform MtzS cells to MtzR. Conversely, rdxA genes from MtzS strains could render MtzR strains to MtzS (45). Thus, rdxA inactivation seemed to be a critical factor for development of resistance in most or all strains throughout much of the world and possible alternative (e.g., plasmid or transposon-specified) resistance mechanisms that might bypass the need for rdxA inactivation seemed uncommon, if they exist at all in any Hp population. This generality seemed

significant in light of apparent geographic differences in Hp gene pools summarized above.

c. MtzS strains differ in ease of conversion to MtzR phenotype: frxA regulation
Although most MtzS Hp strains gave rise to MtzR mutants at frequencies of about 10^{-4} (designated type I strains), others did so only at frequencies of $< 10^{-8}$ (type II strains). Reverse genetic tests involving transformation with DNAs containing null mutant alleles of rdxA and frxA (illustrated in Fig. 5) showed that the critical difference between type I and II strains was whether resistance could be achieved by inactivating just rdxA (type I) or required inactivation of both rdxA and frxA (type II) (45,47,48).
This, in turn, was traced by Northern blotting to differences in levels of frxA transcripts: low in type I; and high in type II (47). Although some 90% or more of MtzS strains from each of several regions (Spain, China, Japan, India, and Peru) were type I (45), it is striking that each of two special mouse colonizing Hp strains tested to date (SS1, X47) is type II (47,48). Recent studies have suggested that inactivation of rdxA and of frxA each impair the ability of these strains to colonize mice (Jeong, Dailidiene. Mukhopadhyay and Berg, unpublished). As a guide to future studies, we propose (i) that these nitroreductase genes can each be important, at least quantatively, in certain human infections, and in addition (ii) that the differences in FrxA nitroreductase levels in type I and type II strains might each also contribute quantitatively to the growth of Hp in particular human hosts or gastric niches.

It is interesting in this context that MtzR derivatives of strain SS1 were selected in mice, and found, in most cases, to be mutant in rdxA (46). Based on the analyses just outlined, we predict that these MtzR SS1 strains also contain mutations in frxA, and perhaps additional mutations as well that allow MtzR Hp to survive in mice. If correct, this would further illustrate the potency of Mtz as a mutagen (point 5, below) and the power of selective forces during chronic infection.

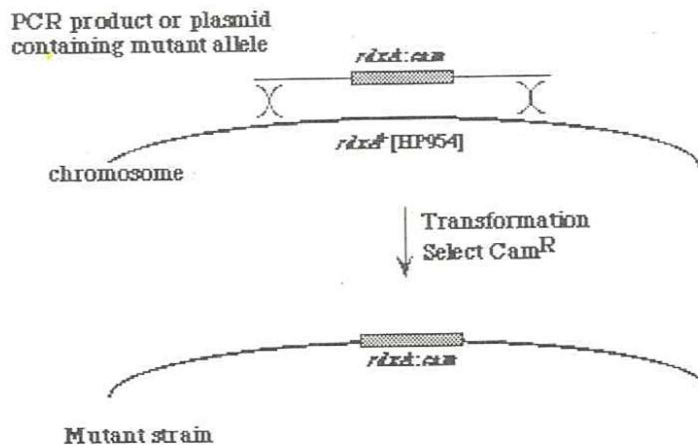

Fig. 5: *Representative DNA transformation with allelic replacement to generate specific mutant strains of Hp. Plasmid DNA or a PCR product containing a mutated allele of the gene of interest (here rdxA mutated by insertion of a cassette encoding resistance to chloramphenicol) is mixed with young exponentially growing Hp cells (with or without enhancement by electric shock; "electroporation"), the mixture is incubated together for several hours or overnight, and then bacteria are spread on medium containing chloramphenicol to select transformants. The structure depicted (the result of recombination with complete replacement of the wild type allele by the engineered mutant allele is easily demonstrated by PCR. This transformation in MtzS type I Hp strains (rdxA active; frxA relatively inactive) results in derivatives with modest level Mtz resistance (MIC of ~32 mkg/ml, up from ~3 mkg/ml). Inactivation of frxA (using, e.g., an frxA-kan knockout mutant allele) results in increased resistance (MIC to ~64 mkg/ml). Inactivation only of frxA in type I strains (rdxA still functional) does not affect their MtzS phenotype. Both rdxA and frxA must be inactivated to achieve a MtzR phenotype in type II MtzS strains. Inactivation of either nitroreductase gene leaves the strain MtzS (if assessed critically by ability of single cells to form colonies on Mtz-containing agar). However, either single mutant derivative of a type II strain may give rise to MtzR derivatives at frequencies about 10^{-4} instead of $<10^{-8}$.*

d. The frxA gene is expressed, albeit weakly in type I strains, and contributes subtly to their phenotype: Inactivation of frxA in type I strains that already contained null mutant alleles of rdxA made them resistant to somewhat higher levels of Mtz (MIC increased from 32 to 64 mkg per ml). Inactivation of frxA by itself, however, did not affect the innate Mtz susceptibility of type I Hp strains with functional rdxA genes. This is attributed to the basal level of FrxA enzyme in type I cells, which can generate bactericidal levels of activation products in cells exposed to high Mtz concentrations (45,48).

e. Mtz activation is highly mutagenic : Growth of Hp on medium with concentrations of Mtz that were close to the MIC of the strain (concentration sufficient to decrease but not eliminate the ability of single cells to form colonies) resulted in an up to several hundred-fold increase in frequencies of rifampicin resistant mutants

(49). This mutagenic effect was evident in MtzR (rdxA and frxA mutant) and MtzS strains grown at Mtz levels near the MIC for the strain. This illustrates that there must be yet additional nitroreductases (distinct from those encoded by rdxA and frxA) that can activate Mtz to a biologically significant level, given sufficient Mtz substrate (49). It may also help explain why some nominally MtzR infections can be eradicated (albeit inefficiently) by Mtz-based anti-Hp therapy. Equally important, frequent subtherapeutic exposure of Hp to Mtz (because of use against other maladies) and its mutagenic effect probably promotes the emergence of Mtz resistance, contributes to the development of resistance to other antibiotics, and speeds bacterial adaptation to individual host defenses and possibly the evolution virulence as well.

f. Mtz induced mutation does not operate on bystanders : We tested if Mtz activation by some Hp cells would cause mutation in adjacent cells, vs. if Mtz is mutagenic only for cells that activate it. A high density mixture of MtzS and MtzR bacteria was grown on medium with a concentration of Mtz that induced mutation in the susceptible strain, but not in the resistant strain. Then the level of newly induced RifR mutants was quantitated in both strain types. We found that this low level exposure to Mtz only stimulated mutation in the MtzS Hp, not in the bystanders (49). Consistent with this, Touati et al (50) found no evidence of Mtz-induced mutation in gastric epithelial cells of H. pylori infected mice. These results can be explained by noting that Mtz is activated by intracellular bacterial enzymes, and that its mutagenic derivatives may be so highly active that they act and are consumed before they can diffuse into neighboring cells (49). This result is comforting, since it indicates that Mtz activation by resident H. pylori strains, although probably speed their adaptation to new circumstances (including exposure to antibiotics), but should not directly affect the evolution of any co-existing bacterial strains nor promote neoplastic transtormation in human epithelial cells to which Hp might be bound.

Technical note: estimation of Mtz susceptibility and resistance : Two considerations indicated that special care is needed when Mtz susceptibility and resistance are to be scored accurately. One consideration stems from the mutagenic effects of Mtz activation just discussed. The second emerged while seeking to resolve disagreements in the literature (51 vs. 45,47), which we (48) interpret as reflecting inadvertent use in ref. 51 of strains that require inactivation of both rdxA and frxA ("type II") to achieve a resistant phenotype, as will be detailed below.

Traditionally drug susceptibility has been determined by monitoring zones of inhibition of growth in dense bacterial lawns (typically starting with at least 10^7 cells per standard petri plate) onto which have been placed filter paper discs containing fixed concentrations of drugs of interest ("disc diffusion"), or "etest" strips that contain concentration gradients of the drug. An alternative method involves monitoring growth vs. non-growth of bacteria after spotting dense suspensions (10^6 cells) on media with different fixed concentrations of drug ("agar dilution"). We could NOT recommend either of these methods for sensitive and reliable scoring of levels of Mtz resistance in a research laboratory setting. Rather, we recommended making a series of ten-fold dilutions of cultures of interest, and then spotting aliquots of each dilution on Mtz-containing and Mtz-free (control) media (ranging from about 100 viable cells to 10^6 cells per spot) (45,48). When MtzR mutants are very rare (e.g., one per 10^7 cells or less) we spread suspensions over larger surfaces and thereby avoid inhibitory effects of bacterial crowding on colony formation. A strain is considered resistant to a given Mtz level only if numbers of colonies on the Mtz-containing and Mtz-free media are equivalent. With media containing higher than MIC levels of Mtz,

additional valuable information comes from estimating survival at each Mtz concentration (45,48).
An application of this rapid titration approach is illustrated in a re-examination of experiments based on agar dilution–based that had led one set of authors (51) to conclude that Mtz resistance results from inactivation of EITHER frxA or rdxA. Our reconstruction experiments (48) showned that that they (51) had used type II strains (requiring inactivation of both rdxA and frxA to achieve a MtzR phenotype, and that inactivation of either gene alone allowed new MtzR mutants to appear at frequencies of approximately 10^{-4}, rather than $<10^{-8}$, as discussed above. Their results can be ascribed to having spotted enough ($>10^{5}$) cells to ensure (in case of frxA or rdxA single gene knockout strains) that each spot would contain significant growth (of new MtzR mutants), which was then scored as full resistance. Tests with more dilute suspensions show that more than 99.9% of cells spotted on such agar were in fact Mtz-sensitive, and that the colonies obtained had been formed by cells that were induced by the Mtz to mutate to a resistant phenotype before they were killed by the Mtz.

Summary and Perspective

Great progress has been made in understanding the population genetic structure and evolutionary forces operating on *H. pylori*, this most diverse of bacterial pathogens; its mechanisms of susceptibility and resistance to antimicrobial agents; and more broadly, its interactions with host during chronic infection and disease. Our studies to date of Hp genotypes in just four regions (East Asia, India, Spain and Peru) stimulate interest in the gene pools of Hp from yet other populations of Eurasia, Africa and the New World. We, in particular, hope to test the possibility that regional differences in predominant Hp genotypes might contribute to the distinctive patterns of gastroduodenal disease in particular human societies. We found that Mtz resistance generally results from loss of function mutations in chromosomal nitroreductase genes, not the acquisition of plasmid- or transposon-borne resistance genes, that even MtzS Hp populations are polymorphic for levels of expression of one of the nitroreductase genes (frxA), and that sublethal Mtz activation can be powerfully mutagenic. These findings provide new cautionary illustrations of how important more prudent antibiotic use can be, and also open up possibilities for developing new better redox-active anti-Hp therapies, for improving our understanding of Hp genome adpation and evolution, and for examining the specificity of bacterial-host interactions during colonization and disease.

Acknowledgment

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PROTEASES OF MALARIA PARASITES: NEW TARGETS FOR CHEMOTHERAPY

Rosenthal PJ, Sijwali PS, Shenai BR, Singh A, Lee B, Gut J
Department of Medicine, University of California, San Francisco, CA, USA

Malaria is one of the most important infectious diseases in the world. *Plasmodium falciparum*, the most virulent human malaria parasite, is responsible for hundreds of millions of illnesses and over a million deaths each year [1]. The treatment and control of malaria is increasingly limited by resistance of malaria parasites to available drugs. New antimalarial therapies, ideally directed against new targets, are needed [2].

Plasmodial proteases hydrolyze hemoglobin in an acidic food vacuole to provide amino acids for parasite protein synthesis [3,4]. In studies of cultured *P. falciparum* parasites, cysteine protease inhibitors blocked hemoglobin hydrolysis, causing the accumulation of undegraded hemoglobin in the food vacuole and a halt in parasite development [5]. We are now characterizing plasmodial cysteine proteases and testing cysteine protease inhibitors as potential new antimalarial agents.

Three papain-family cysteine proteases, or falcipains, are expressed by *P. falciparum* trophozoites. Falcipain-1 was the first gene identified, but characterization of this protease has been limited by an inadequate system for heterologous expression, and its function remains uncertain [6]. Falcipain-2 [7] and falcipain-3 [8] have now been expressed in good yield in *E. coli* and the proteins refolded to active enzymes, allowing detailed biochemical characterization. Both falcipain-2 and falcipain-3 appear to function as hemoglobinases in the parasite food vacuole. They each have acidic pH optima, require reducing conditions for maximal activity, prefer peptide substrates with Leu at the P_2 position, and were localized to a *P. falciparum* vacuolar fraction. Falcipain-3 differs from falcipain-2 in that it undergoes efficient processing to an active form only at acidic pH, is more active and stable at acidic pH, and has much lower specific activity against typical papain-family peptide substrates. However, both falcipain-2 and falcipain-3 exert similarly potent hemoglobinase activity under physiological conditions. Falcipain-2 is unique among studied papain-family enzymes in that the pro-domain is not required to mediate folding to active enzyme [9]. Specific features of the plasmodial proteases likely have relevance to the specific functions of the different enzymes.

We have recently also characterized a related protease of the rodent malaria parasite *P. vinckei,* named vinckepain-2 (A. Singh, unpublished). This enzyme is also a potent hemoglobinase, but it has some important differences from the falcipains in fine substrate and inhibitor sensitivity. Notably, the enzyme and homologs from other rodent malaria parasites have an unusual amino acid at a site that is highly conserved among papain-family enzymes (Gly_{23} is replaced by Ala). Vinckepain-2 has an unusual preference for substrates with P_1 Gly, but mutagenesis to replace Ala_{23} with Gly (the amino acid found at this location in most papain-family proteases) led to marked changes, including loss of the preference for substrates with P_1 Gly and markedly greater catalytic activity.

Multiple classes of cysteine protease inhibitors have antimalarial activity. In general, compounds block the hydrolysis of hemoglobin and development by cultured parasites at similar concentrations, providing target validation. Optimal compounds, including peptidyl fluoromethyl ketones and vinyl sulfones, inhibited parasite development at nanomolar concentrations and cured mice of otherwise lethal *P.*

vinckei infections [10-13]. A number of classes of inhibitors were equally active against *P. falciparum* strains that differ markedly in sensitivities to established antimalarial drugs [14]. We are now testing a number of classes of protease inhibitors against multiple plasmodial cysteine proteases in efforts to both better characterize the biological roles of falcipains and to identify optimal antimalarial agents.

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DEVELOPMENTS OF LABORATORY DIAGNOSIS IN AMEBIASIS
AMEBİASİSİN LABORATUVAR TANISINDA GELİŞMELER

Tanyüksel M
GATA Tıbbi Parazitoloji Bilim Dalı, Etlik, Ankara, TURKEY

Summary
In this review, the importance of microscopy, culture/isoenzyme analysis, antigen/antibody detection-based methods and PCR are described in the diagnosis of amebiasis. In addition, use of molecular assays (PCR-based) are extremely powerful tools for genetic differences are discussed.

Özet
Bu derlemede, amebiasisin tanısında önemli bir yer tutan mikroskopi, kültür/izoenzim analizi, antijen ve antikor saptanmasına yönelik testler ile PCR'ın önemi anlatılmaktadır. Ayrıca, PCR-temelli moleküler yöntemlerin kullanımının genetik farklılığın araştırılmasında oldukça yeterli araçlar olduğu tartışılmaktadır.

Giriş

Amebiasis, etken *Entamoeba histolytica*'nın insanlarda oluşturduğu asemptomatik taşıyıcılıktan barsak ülserasyonları ve karaciğer abselerine kadar geniş bir klinik yelpazede, özellikle gelişmekte ve az gelişmiş ülkelerde görülen yaygın bir protozoer infeksiyondur. Sözkonusu amip, ilk kez 1869'da cerrah Timothy Lewis tarafından koleralı bir hastanın dışkı örneğinden izole edilmiştir. Robert Koch, amebiasisin patogenezini araştırmak amacıyla dizanterili ve karaciğer abseli iki hastanın otopsisisinde, kolon ülserlerin tabanında ve karaciğer abselerinin kapillerlerinde amipleri yoğun olarak gözlemiştir. Couincilman ve Lafleur Baltimore'da 14 olguluk amebiasis serisinde kolonda şişe dibi görünümlü amebik ülserlerini tanımlamış, *amebik dizanteri* ve *amebik karaciğer absesi* terimleri de bu saye ile tıp literatürüne girmişlerdir [1].

E. histolytica dünyada yaklaşık olarak 50 milyon insanı infekte eden ve 100 bin insanın da ölümüne yol açan acil enfeksiyonlar içinde değerlendirilen önemli bir halk sağlığı sorunudur [2].

Son yıllarda iki önemli gelişme amebiasisde dönüm noktası olmuştur: antijene dayalı duyarlı ticari test kitlerinin varlığı.ve *E. histolytica* ile *E. dispar*'ın ayrılabilmesi / klinik farklılıklarının vurgulanması.

Her ne kadar *E. histolytica* ile *E. dispar*'ın mikroskobik ayrımı mümkün olmasa ve morfolojik benzerlikleri bulunsa da bugün için her iki tür arasında biyokimyasal, immünolojik ve genetik farklılıkların bulunduğuna dair geçerli bilgiler elde edilmiştir [3-5].

Her iki tür için a) Farklı DNA restriksiyon fragment patternleri, b) Tekrarlayıcı DNA sekanslarının tek olması, c) Genomdaki aktin geni organizasyonunun farklı olması gibi DNA seviyesinde de farklılıkları bulunmaktadır [5, 6].

İmmünolojik ve antijenik farklılıklar, monoklonal antikorların kullanımı ile gözlenebilmektedir. Diğer bir önemli fark, *E. histolytica*'da oluşan humoral immun yanıtın *E. dispar*'da gözlenmemesidir [7].

Mikroskopi

Klasik ve yaygın olarak *E. histolytica* tanısı mikroskopi ile konmaktadır. Ancak, *E. dispar* ile morfolojik benzerlikleri nedeniyle mikroskobik ayrımları çok zordur. Dolayısıyla sitolojik tanının duyarlılığı ve özgüllüğü düşüktür. *E. dispar*'ın dünyada sıklığının *E. histolytica*'dan daha fazla olması (10/1) nedeniyle ayrımların yapılması tanı ve tedavinin yönlendirilmesinde önem arz etmektedir. *E. dispar*'lı bazı olgularda mikroskopide eritrosit içeren amiplerin azımsanmayacak sayıda olması (%16), mikroskopinin hiç de güvenilir bir yöntem olamadığını göstermektedir [8]. Sıklıkla, fekal lökositler *E. histolytica* kistleri, makrofajların da trofozoitler ile karışması göz ardı edilmemelidir. Mikroskopinin düşük duyarlılığı yüzünden bu enfeksiyonların %50'sinin kaçırılma riski bulunmaktadır.

Kültür/ Zimodem Analizi

Zimodem analizine dayalı *E. histolytica* ile *E. dispar*'ın ayrımı yapılabilmektedir. Bununla birlikte, dışkı kültürünü takiben uygulanan zimodem analizi daha duyarlı bir yöntem olmasına karşın klinik ve pratik uygulamalardaki zorluklar ve daha fazla zaman alması dezavantajları yaşanmaktadır.

Zimodem analizine göre, hexokinase izoenzime patternlerinin belirlenmesi oldukça yararlıdır. Bu teknikle, yavaş göç eden bantların varlığı *E. dispar*'ı, hızlı göç eden bantların varlığı *E. histolytica*'nın varlığını ortaya koymaktadır. Bir çalışmada, tropikal ve subtropikal bölgelere seyahat eden 60 Alman turistinin 18'inde hem hexokinase izoenzimi hem de PCR analizi ile *E. histolytica* ile infekte olduğu bulunmuştur [9]. Bangladeş'te endemik olarak bilinen Dakka, Mirpur bölgesinde yapılan seri çalışmalarda kültür / zimodem analizi sonuçları, PCR ve antijen dayalı ELISA testleri ile oldukça uyumlu olarak elde edilmiştir [10, 11].

Ancak son zamanlarda da izoenzimlerinden bazılarının bakteri kontaminasyonlarından kaynaklanan sorunlar nedeniyle bugün için bilinen 20 zimodemden aslında 3'nün geçerli olması gerektiğine dair görüşler bulunmaktadır (L. Diamond ve G. Clark kişisel görüşme).

Antijen Saptamaya Yönelik Testler

Dışkıda antijen saptamaya yönelik birçok kit halihazırda ticari olarak mevcuttur. Ancak bunların birbirlerinden ayrımları mevcuttur. İdeal antijene dayalı test kiti özellikle erken enfeksiyon safhasında yüksek duyarlılık ve özgüllüğe sahip olmalıdır. Halihazırda piyasada bulunan ticari kitlerden Merlin Optimun S antijen kiti sisteinden zengin *E. histolytica* proteinine (SREHP) karşı monoklonal antikorların, Alexon kiti *E. histolytica* karşı poliklonal antikorların, TechLab kiti de *E. histolytica* lectine karşı monoklonal antikorlara karşı özgüllüğünü göstermektedir. Ancak bunlar içinde *E. histolytica* lectin antijenik yapısına uygun olan kitlerle daha duyarlı sonuçlar elde edilmiştir. Lectin, *E. histolytica*'nın 170-kDa'luk immunodominant antijenik molekülü olup, sadece tanı amaçlı değil aynı zamanda aşı çalışmalarında da kullanılan önemli bir yüzey adhesin faktörüdür. Bazı araştırmacılar, TechLab *E. histolytica* antijen kitini *E. histolytica* enfeksiyonlarını saptamada zimodem analizine ek olarak altın standart olarak kabul edilen kültür yönteminden daha duyarlı bulmuşlardır [11].

Serumda *E. histolytica*'ya bağlı amebik karaciğer abseli (AKA) olgularda erken dönemde anti-lectin antikorlarının saptanması oldukça önemlidir. Yine Dakka, Bangladeş'te yapılan bir çalışmada, tedavi öncesi AKA'lı hastaların %94'ünde (15/16) antikorlar saptanabilirken, 7 günlük metronidazol ile tedavi sonrasında bu yöntemle duyarlılık %16 (3/19) olarak bulunmuştur (Haque R ve Petri WA, basılmamış bilgi).

Son yıllarda tükrükte invaziv hastalığın tanısına yönelik çalışmalarda (lectin varlığını ortaya koymada) erken enfeksiyonlarda yüksek duyarlılık (%97.4) ve orta derecede (%65.8) özgüllük elde edilebilmiştir [12].

Antikor Saptamaya Yönelik Testler

Endemik alanlarda çok kez *E. histolytica* enfeksiyonlarına maruz kalanlar çoğunlukla asemptomatik amebiasis tablosu oluşmakla beraber çapraz reaksiyonlar nedeniyle tanı koymada sorunlar yaşanmaktadır. Bir diğer sorun da, uzun süren özellikle IgG yüksekliğine bağlı antikorların akut ya da kronik enfeksiyon ayrımında zorluk yaşanmasıdır. Ne yazık ki, anti-amebik antikorlar bazı hastalarda konak immünitesi ve parazitin virulansına bağlı olmak üzere AKA gibi invaziv ilerleyici prognoza gidişi önleyememektedir [13]. Asemptomatik ve kist taşıyıcılarında ve kronik non-dizanterik amebiasis tablolarında antikorların yokluğu ya da yetersizliği diğer bir önemli sorundur. Geçirilmiş enfeksiyonlar sonrası uzun süreli antikor stabilitesi sonuçları etkileyebilmektedir. Bugüne değin indirekt hemagglutinasyon (IHA), karşılıklı elektroforez (CE), amebik jel difüzyon test, kompleman fiksasyon test (CF), indirekt fluoresan antikor (IFA), lateks agglutinasyon (LA) ve enzyme-linked immunosorbent assay (ELISA) gibi testler kullanılagelmiştir.Bunlar içinde AKA hastaların değerlendirilmesinde ELISA ve LA, IHA ve CF testlerine göre daha duyarlı ve değerli testler olarak kabul edilmektedirler. ELISA düşük maliyet, hızlılık ve yüksek duyarlılık ve özgüllük gibi nedenlerle daha çok tercih edilen bir serolojik yöntemdir [14]. Kraoul ve ark bir çalışmada, anti-amebik antikorların saptanmasında duyarlılıkları ve özgüllüklerini IHA için %97.6-%97, LA için %95-%95 ve ELISA için %93-%100 olarak bulmuşlardır. Maliyetleri test başına IHA 3.78 Amerikan Doları, LA 4.88 Amerikan Doları ve ELISA için 5.10 Amerikan Doları olarak hesaplanmıştır [15]. Cummins ve ark başka bir çalışmada, IFA testinde 1/80 titrede %20 gibi yalancı pozitiflik ve tedavi sonrası 8 ay-2 yıl kadar süren antikorların varlığı gibi bir sonuçla karşılaşmışlardır [16]. Tekrarlayan amebiasis olgularında tükrükte IgA aranmasına yönelik testlerin, düşük antikor seviyelerinde rastlanan IgG ve IgM türü antikor handikaplarında yararlı oldukları bulunmuştur [17, 18]. Epidemiolojik çalışmalarda, DNA problarının kullanımının da yararlı olduklarına ilişkin çalışmalara rastlanılmıştır [19, 20].

PCR

Son yıllarda moleküler ve hücresel biyoloji alanındaki gelişmelerin anlaşılmasında, tanı, epidemiolojik prevalansın değerlendirilmesinde uygulama olanakları geliştirilmiştir. Moleküler tanı yöntemlerinin uygulanması da yaygınlaşmıştır. Bunlardan biri olan PCR metodu *E. histolytica*'nın tanımlanmasında hızlı, daha duyarlı sonuçlar vermesi dışında, olası dışkı lökositleri, makrofajlarından kaynaklanan mikroskobi hatalarını da aza indirmektedir [21]. Bu yöntemle, *E. histolytica* kistlerini 4°C'de 1 ay sonra bile PCR ile yakalama şansımız bulunmaktadır [22]. ELISA yöntemlerine göre daha duyarlı oldukları istatiksel olarak da gösterilmiştir [21]. Maliyetinin yüksek ve daha fazla zaman alması dezavantaj olarak değerlendirilmektedir. Yeterince eğitimli personel ve ekipman olmayışları nedeniyle gelişmekte olan ülkelerde çok da kullanışlı olmadığı öne sürülmektedir [23]. Ancak halihazırda tanının doğrulanması ve tür tayini çalışmalarında ELISA'ya karşı üstünlükleri göz ardı edilemez. Belki daha da önemlisi epidemiyolojik verilerin değerlendirilmesi, türler arası varyasyonların belirlenmesi, tanı kitleri ve aşı çalışmalarının geliştirilmesinde çok önemli bir yere sahiptir.

Tablo 1: Amebiasis Tanısında Testlerin Duyarlılık Ve Özgüllükleri (Kolit Ve Karaciğer Abseleri) [24]:

Test	Kolit		Karaciğer abseleri
	Duyarlılık	Özgüllük	Duyarlılık
Mikroskopi (dışkı)	< 60%	10-50%	<10%
Mikroskopi (abse mayii)	?	?	< 25%
Kültür/ izoenzim analizi	PCR test ya da antijen ELISA'dan dütük	Altın standart	<25%
Dışkıda antijen saptama (ELISA)	>95%	>95%	Genellikle negatif
Serumda antijen saptama (ELISA)	65% (erken dönem)	>90%	~13% (geç dönem) ~100% (ilk 3 gün)
Absede antijen saptama (ELISA)	?	?	~100% (Tedaviden önce)
Tükürükte antijen saptama	?	?	70%
PCR (dışkı)	>70%	>90%	?
Serumda antikor saptama (ELISA)	>90%	>85%	70%-80% (akut) >90% (kronik)

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MALARIA VACCINES

Ok ÜZ
Department of Parasitology, Faculty of Medicine,
Celal Bayar University, Manisa, Turkey

Why is a malaria vaccine needed?

Malaria is an infection caused by four plasmodia that infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*. The infection is endemic in 92 countries (1) and has been estimated to represent 2.3% of the overall global disease burden and 9% in Africa (2). About 41% of the world's population is at risk, and 400 million clinical cases are yearly reported (3). Over two million people per year are died due to malaria, mainly among children in sub-Saharan Africa. The emergence of drug resistant parasites, the increase in insecticide-resistance of the vectors, and the potential change in the distribution and incidence of malaria due to global warming emphasize the urgent need for the development of a safe and effective vaccine as a cost-effective addition to currently available malaria control.

How does the human immune response interacts with the parasite?

It is important to understand the pathogenesis of the infection, the life cycle of the parasite and the interaction between the human immune response and the parasite.

1-Pre-erythrocytic Stage: This stage consists of sporozoite and liver stages. The sporozoite is inoculated into the skin during an infected anopheline mosquito's bite. Within minutes of entering the blood stream, it penetrates inside the hepatocytes. High titer long lasting specific antibodies targeting the sporozoites can be effective in this stage. Nevertheless, every sporozoite must be neutralized rapidly, if even only one sporozoite evades the protection, it may amplify the infection by producing large numbers of merozoites (up to 40,000) in hepatocyte, and each merozoite is capable of invading a red blood cell. A cell-mediated immunity (mainly cytotoxic T lymphocyte response) is thought to be critical during the liver stage (4). Some sporozoites of *P. vivax* and *P. ovale* become hypnozoites which may cause relapses (5).
2-Asexual blood stage: Merozoites begin cycles of invasion, replication and rupture, in red blood cells every 48 or 72 hours. The invasion of new cells takes only a few seconds and then the parasite is hidden within the red blood cell. Antibody-dependant cell-mediated cytotoxicity and complement lysis might be effective in this stage (4).
3-Sexual stage: Some intra-erythrocytic merozoites develop into the sexual forms (gametocytes). When infectious gametocytes are taken up by a mosquito with a blood meal, they mature to form gametes and fertilize one another to produce zygote, ookinete and infective sporozoites, respectively (5). The host's antibodies transfer into anopheline mosquito during the blood meal and may prevent sporozoite formation by neutralizing sexual stages.

Who needs the malaria vaccine and how many malaria vaccines are needed?
Risk groups among those living in malaria endemic areas are infants, children, and pregnant women. Malaria-naive travelers, susceptible to severe disease after acquiring their first infection, constitute another risk group. The *P. falciparum* parasite deserves most attention because of the variety and severity of disease syndromes that it causes (6).

Candidate vaccines are based on various antigens or combinations of antigens derived from different stages of the parasite.

Pre-erythrocytic stage vaccines are anti-infection vaccines designed to prevent all manifestations of the disease, and might be most useful to malaria-naive people and military personnel spending brief periods in endemic countries or residents of low endemic areas.

Asexual blood-stage vaccines would not prevent people from getting infected, but the severity and lethality of the disease would be reduced. These vaccines are also called as anti-morbidity, anti-disease, or anti-mortality vaccines and might be most appropriate for people, especially children and pregnant women, living in endemic areas. Even only partial protection, limiting peak asexual parasitemia, may dramatically reduce morbidity and mortality in Africa, where other control modalities have not been effective.

Sexual blood stage vaccines are also called as transmission blocking or anti-mosquito-stage vaccines which would only prevent the mosquitoes from spreading malaria to new hosts, and could contribute to eliminating the infection. These vaccines would not directly benefit the vaccinee, therefore should be combined with an asexual blood-stage vaccine. A fully effective pre-erythrocytic stage vaccine that would presumably prevent appearance of all asexual blood-stage parasites including the gametocytes, would obviate the need for a sexual blood stage vaccine (4,6).

Based on the experience and information gained during the studies on *P. falciparum* vaccine, progress is also being made to develop a *P. vivax* vaccine (7).

It seems difficult to achieve a protection against several species with a single vaccine. Once successful vaccines have been developed against *P. falciparum* and *P. vivax*, it should be easier to create similar vaccines for *P. malariae* and *P. ovale*.

Why has a malaria vaccine not been developed?

There are many potential explanations for the lack of current success. The parasite expresses different proteins at different stages of its life cycle (8), a vaccine directed to liver-stage parasites may not inhibit the growth of blood-stage parasites (6).

Culture of infective sporozoites is impossible and production of sufficient quantities of blood-stage parasites for vaccination purposes is not feasible. Appropriate *in vitro* assays for predicting protection are also difficult to identify and validate (9).

Only a few parasite proteins can not duplicate the immunity elicited by exposure to a parasite which has thousands of proteins (10).

Over 5,000 candidate proteins and multiple variants of many of these have been identified, but there is no validated models that reliably predict the protection value of a vaccine formulation in humans. Primate models may assist antigen selection, but the development of useful formulations and vaccine regimens will require several human phase 1/2 trials, which is not an easy work. It is also not easy to obtain a strong and long-lasting immune response in humans, even though the same preparation is often strongly immunogenic in test animals (6).

The public support for development of malaria vaccines is roughly tenfold less than the support for a vaccine for HIV — a disease of comparable global impact (6).

Why a malaria vaccine is feasible?

Immunization of experimental animals and human volunteers with live irradiated sporozoites induces protection against subsequent challenge with virulent sporozoites (9,10).

Naturally acquired immunity is developed by the people infected repeatedly by malaria. Children who survive to the age of 7–10 rarely develop life-threatening *P. falciparum* infections in hiperendemic areas of Africa (10).

Sera from animals immunized with prototype human transmission-blocking vaccines have shown success in protecting mosquitoes against infection by *P. falciparum* and *P. vivax* (6).

These results suggest that the efficacy of all three types of vaccines (sporozoite, blood, and mosquito stages) has been proved in principle.

What are the strategies for vaccine design?

Antigens that meet specific criteria should be chosen.

Antigens from different stages should be combined.

Several antigens from a single stage should be combined.

Vaccines should be as simple as possible.

The right kind of immune response must be induced (6).

What about clinical trials?

A series of phase 1/2 vaccine trials has been reported using synthetic peptides or recombinant proteins based on malarial antigens mostly directed against sporozoites or liver stages, in last 15 years. Protein-based vaccines are promising, but alternative technologies such as DNA-based vaccines are considered to be revolutionary due to their ease of production, storage, low cost, long shelf life, and ability to induce good T-cell responses and to present several antigens from different stages with a single injection (6,9).

Only a few *P. falciparum* vaccines have been tested in field trials. The most well-known is SPf66, a synthetic peptide vaccine, directed at blood-stage parasites. The vaccine caused a modest reduction of malaria attacks in initial trials in South America and Tanzania, but subsequent trials in Gambia, Tanzania, Thailand and Brazil showed no evidence of efficacy (6,9). There are two other promising vaccines that have recently undergone field trials. RTS,S, a preerythrocytic vaccine based on circumsporozoite surface protein fused to HbsAg, was found to be significantly effective in protection against natural *P. falciparum* infection in semi-immune adult man in the Gambia, although the protection was not long-lived (11). The other vaccine is a combination of three asexual blood-stage recombinant antigens (MSP1, MSP2, RESA), and has been tested in children in Papua New Guinea. The trial resulted in significant decrease in the parasite density (6).

A DNA vaccine including a string of CTL epitopes from six different *P. falciparum* pre-erythrocytic antigens has been in clinical evaluation in Oxford and in The Gambia with encouraging results (12). DNA plasmids encoding malaria antigens have generated antibody responses in animal models but not in humans as yet (6).

Most promising *P. vivax* vaccine candidates are *P. vivax* circumsporozoite protein (CSP) and merozoite surface protein 1 (MSP1) (7).

When will there be an effective malaria vaccine?

Many projects are going on for maximizing magnitude, quality and longevity of protective immune responses for a multistage, multiantigen vaccine. New approaches to producing modified antigens are developed, together with new technologies such as DNA vaccines and novel adjuvants, but it is not easy to predict when an effective product will be available. It will still be many years from now until sufficient efficacy is obtained.
There is a need for collaborative work to achieve the goal of an effective malaria vaccine. Some of the research organizations, foundations, national funding agencies and drug companies working for this goal:

Malaria Vaccine Initiative at the Program for Appropriate Technology in Health (through a grant from the Bill & Melinda Gates Foundation)
US Agency for International Development's (USAID) Malaria Vaccine Development Program (MVDP)
European Malaria Vaccine Initiative
Multilateral Initiative on Malaria in Africa (MIM)
African Malaria Vaccine Testing Network (AMVTN)
US NIH Malaria Vaccine Development Unit
World Health Organization (WHO)
Tropical Diseases Research (TDR)
US Department of Defense
Roche
Wellcome Trust
GlaxoSmithKline
Institut Pasteur

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MOLECULAR MARKERS OF ANTIMALARIAL DRUG RESISTANCE IN UGANDA

Rosenthal PJ[1], Kamya MR[2], Dorsey G[1], Staedke SG[1], Gasasira A[2], Njama D[2], Sendagire H[2], Lamola S[1], Kyabayinze D[2]
[1]Department of Medicine, University of California, San Francisco, CA, USA
[2]Departments of Medicine and Biochemistry, Makerere University, Kampala, UGANDA

Antimalarial drug resistance

Antimalarial drug resistance is a large and growing problem, especially in Africa [1,2]. Chloroquine (CQ) remains the drug of choice for uncomplicated falciparum malaria in most countries, but it increasingly fails as effective therapy. In a few countries the antifolate combination sulfadoxine/pyrimethamine (SP, Fansidar) has replaced CQ as first-line therapy for uncomplicated malaria, but increasing resistance to this therapy has also been noted [3,4]. A number of other therapies are available for malaria, but in developing countries these may be limited by cost, toxicities, or resistance. More data is needed to best identify appropriate new antimalarial therapies for Africa. Also, additional studies are needed to determine the best means of evaluating antimalarial drug efficacy and to utilize new molecular tools for the characterization of malaria.

Table 1: Results of clinical trials.

Trial	Drugs	Resistance (%)		Notes
		Clinical	Parasitol.	
CQ [5]	CQ	47	70	Children and adults; Risk factors for resistance: young age, fever, recent CQ use
CQ vs. SP [6]	CQ SP	54 11	72 30	Children and adults; SP significantly more efficacious than CQ
AQ vs. SP vs. AQ/SP [11]	SP AQ AQ/SP	10 7 3	26 16 10	Children and adults; AQ/SP significantly more efficacious than SP
SP vs. CQ/SP vs. AQ/SP	SP CQ/SP AQ/SP	15 7 0	30 17 1	Children and adults; randomized; blinded; AQ/SP significantly superior to both SP and CQ/SP
SP vs. AQ/SP vs. AS/SP [12]	SP AQ/SP AS/SP	18 1 1	32 2 5	Longitudinal study; Children <5; randomized; 577 episodes; combinations offered significantly improved efficacy compared to SP alone; SP/AQ superior to SP/AS

Clinic-based studies of antimalarial drug resistance in Kampala

We have conducted a series of studies of antimalarial drug efficacy in Kampala, Uganda. For four clinic-based studies, patients with uncomplicated malaria were

treated with different therapies, and outcomes were followed by standard protocols, with clinical evaluations on days 1, 2, 3, 7, and 14, and repeat thick blood smears on days 3, 7, and 14. Patients who did not return for follow-up were visited at home. Primary outcomes were based on established parasitological (S, RI-III) and clinical (adequate clinical response (ACR), late treatment failure (LTF), early treatment failure (ETF)) criteria (Table 1). Our studies have shown that CQ resistance is very common, and that this treatment is no longer an acceptable therapy for malaria in Uganda [5]. The antifolate combination SP was much more effective than CQ for uncomplicated malaria, but resistance rates to this therapy were higher than expected [6]. The 4-aminoquinoline amodiaquine (AQ), which is closely related to CQ, has recently been shown to have good efficacy against CQ-resistant malaria, and to have lower risks of toxicity than previously believed [7-10]. In our first study of AQ, the drug was effective (with success rates equivalent to those of SP), and no serious toxicities were seen [11]. This study also provided the first evaluation of the combination of AQ and SP, and this regimen offered 97% clinical efficacy [11]. Lastly, the combination of CQ/SP, which has been advocated as a replacement therapy for Uganda, was clearly inferior to AQ/SP, and offered relatively little advantage over SP monotherapy (Gasasira, A., in preparation). In summary based on these clinical studies, CQ resistance is unacceptably high in Kampala, resistance to SP is much lower, but concerning, and the combination of AQ/SP may offer an affordable, highly effective new therapy for the treatment of uncomplicated malaria.

Longitudinal study of antimalarial drug resistance in Kampala

To better characterize the full impacts of different antimalarial therapies, we recently completed a longitudinal comparison of the efficacies of different therapies over the course of multiple treatment episodes [12]. This study included the regimens discussed above, and also artesunate (AS), a promising new drug derived from artemisinin (also known as qinghaosu) that is highly efficacious, but limited by high cost and reports of recrudescence after therapy. Healthy children, aged 6 months to 5 years, were recruited from Kampala and randomized to three different treatment regimens (SP/placebo, AQ/SP, or AS/SP) for all future episodes of uncomplicated malaria (for each child, the same regimen was used to treat each episode). Treatment outcomes were assessed over 14 days using standard parasitological and clinical criteria. Considering each malaria episode independently, after 14 days, clinical and parasitological failures were much more common in the SP treatment group (Table 1). Both combination therapies offered markedly improved short-term efficacy over SP monotherapy. Side effect profiles between treatment arms were comparable, and no major adverse events were identified. Considering outcomes beyond 14 days, the risk of needing repeat treatment within 4 weeks was 28% in the AS/SP group compared to 12% in the AQ/SP group. Treatment incidence densities (total number of treatments per person year of follow-up) also differed significantly (4.3/year for SP, 3.6/year for AS/SP, and 2.8/year for AQ/SP; Figure 1). Thus, the combinations decreased the average number of treatments, and the inexpensive regimen of AQ/SP had markedly greater efficacy in this regard than either SP or AS/SP. Furthermore, the longitudinal study demonstrated that, in contrast to standard WHO recommendations, comparisons of antimalarial treatment efficacies must be carried out for at least 4 weeks to avoid the underappreciation of late treatment failures.

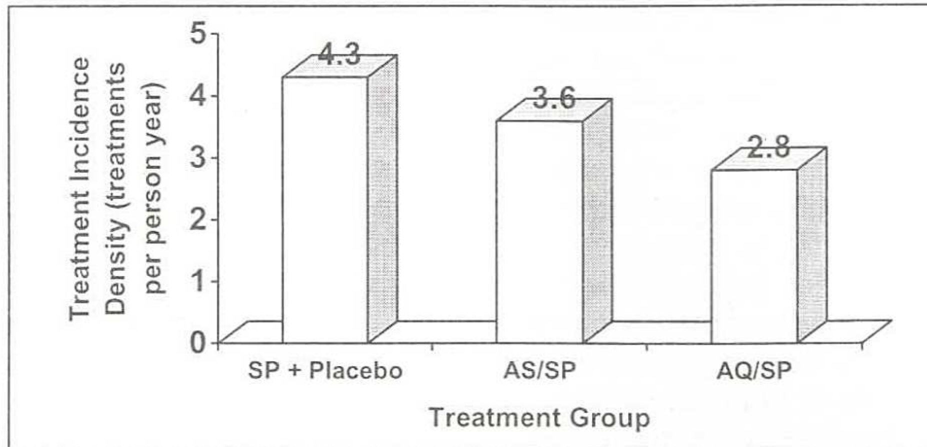

Figure 1: Longitudinal comparison of treatment efficacies. Differences between the treatment groups were statistically significant.

Molecular markers of CQ resistance

Recent studies have identified mutations in the *P. falciparum* gene *pfcrt* as a key mediator of CQ resistance [13,14]. Mutations in *pfmdr-1* may also contribute to resistance. Samples from our first study, which evaluated the efficacy of CQ, were analyzed for mutations in *pfmdr-1* and *pfcrt*. Blood samples were stored on filter paper, and parasite DNA was extracted with chelex. Samples were analyzed by mutation-specific PCR and restriction endonuclease digestion techniques designed to identify specific mutations of interest. All of 114 studied isolates had the *pfcrt* K76T mutation that has been strongly linked to in vitro CQ resistance [15]. Analysis of 7 additional *pfcrt* mutations and two *pfmdr-1* mutations identified no correlations between genotypes and treatment outcomes. Our results were consistent with other findings showing very high prevalence of the K76T mutation in many areas, and demonstrated that the CQ resistance K76T *pfcrt* mutation is already deeply entrenched in Kampala. The study also proved that the molecular evaluation of clinical samples collected from filter paper, often after storage for many months, was readily achievable.

Molecular markers of SP resistance

SP is a combination of the DHFR inhibitor sulfadoxine and the DHPS inhibitor pyrimethamine. We are currently evaluating samples from SP-treated patients in our second clinical study for mutations in the *dhfr* and *dhps* genes that are known to mediate resistance to SP. DHFR analysis using nested PCR and restriction endonuclease digestions has been completed for a subset of samples. DHFR mutations previously shown to mediate pyrimethamine resistance were very common in parasites evaluated before the onset of therapy (85/91 [92%] S108N; 84/91 [92%] N51I; 38/91 [42%] C59R). Unusual mutations, predicting higher level resistance (1/91 S108T with A16V; I164L seen in one follow-up specimen), were rare, but are concerning as predictors of worsening resistance. DHPS analysis is ongoing; mutations in codons 437 and 540 appear to be common. Infections by parasites with fully wild-type DHFR were all successfully treated with SP. Our interim results indicate that resistance-mediating polymorphisms in DHFR and DHPS are common in Uganda, providing further support that SP monotherapy will not be an adequate replacement for CQ for the treatment of uncomplicated malaria in Uganda.

Molecular fingerprinting to distinguish recurrent and new infections after drug failure

Our longitudinal analysis provided a valuable assessment of the long-term efficacy of antimalarial therapies and also raised interesting questions. Importantly, it was unclear whether differences in the rates of late (after 14 days) failures of some regimens were due principally to late recrudescence of infecting parasites or to differential rates of reinfection. To better evaluate outcomes, we therefore modified recently developed techniques to fingerprint *P. falciparum* isolates. Of 577 episodes of malaria in our study population, 386 were appropriate for analysis, in being linked to (i.e. occuring before or after) another episode in the same individual and not complicated by other factors (e.g. very early treatment failure, non-falciparum malaria infection). The genotypes of consecutive infecting strains were evaluating by extracting DNA from filter paper samples and evaluating polymorphisms in the MSP-1 gene. Techniques for MSP-1 analysis utilized recently established nested-PCR techniques. Episodes were classified as treatment failures if the subsequent infecting strain revealed a complete or partial match with the initial strain, and as new infections if the subsequent strain revealed a completely new pattern. Evaluations were also done for two other genes (MSP-2 and GLURP), using similar techniques, but analysis of only MSP-1 provided the most meaningful analysis, and was considerably simpler than the 3-gene analysis. Treatment failure rates after reclassification based on MSP-1 genotyping showed that reinfection rates did not differ between treatment groups but that recrudescences were more prevalent with SP and AS/SP than with AQ/SP, and that these recrudescences often occurred 14-28 days after the onset of therapy. This study offered additional support for our conclusion that a 14 day follow-up period is inadequate for the analysis of antimalarial drug sensitivity. In addition, the study indicated that the AS/SP regimen, which is highly touted as a new treatment for malaria in Africa, suffers from frequent late failures (after reclassification based on genotyping the recrudescence (or true drug failure) rate at 28 days was only 4% for AQ/SP, but 17% for AS/SP and 31% for SP monotherapy). Thus, the AS/SP regimen must be reconsidered as an effective therapy for malaria in East Africa. In contrast, the AQ/SP regimen led to very few late recrudescences, and remains a promising and affordable antimalarial regimen for Africa.

Summary

Clinical studies in Kampala and related molecular studies have allowed the following conclusions. (a) CQ resistance is extremely common in Kampala.(b) SP is far superior to CQ in efficacy for uncomplicated malaria, but increasing resistance to this drug is concerning. (c) AQ showed surprisingly good efficacy and minimal toxicity. An AQ/SP combination regimen is very promising, as it provided nearly 100% efficacy for the treatment of acute uncomplicated malaria and, upon longitudinal analysis, it offered significant benefit compared to either SP or an AS/SP combination. Despite concerns related to past experience with chronic chemoprophylaxis in non-Africans, we identified no major toxicities from AQ after ~500 treatments. (d) A longitudinal study showed that clinical outcomes must be assessed for at least 4 weeks after treatment for uncomplicated malaria. (e) Known mutations that mediate CQ and SP

resistance are common in *P. falciparum* isolates from Kampala, supporting studies of other therapies. (f) Molecular genotyping methods offer a simple means of improving the interpretation of clinical trials. Additional clinical evaluations of antimalarial drug efficacy and related molecular studies are ongoing.

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ISOTHERMAL DNA AMPLIFICATION—RAMIFICATION AMPLIFICATION

Li H, Zhang W, Yi J, Zhang DY

Department of Pathology, Mount Sinai School of Medicine

New York University, New York, New York

Abstract

We have developed an isothermal DNA amplification method termed ramification amplification (RAM). This method was designed to utilize a sequence-special circular probe (C-probe) that forms a closed cycle by a DNA ligase when the C-probe hybridizes onto a target. In the presence of excess primers, a DNA polymerase extends the bound forward primer along the C-probe and displaces the downstream strand, generating a multimeric single-stranded DNA (ssDNA). The mechanism of the reaction is similar to "rolling circle" replication of bacteriophages *in vivo*. The resulting multimeric ssDNAs then serves as a template for multiple reverse primers to hybridize, continuous extension and displacing downstream DNA. These processes generate large ramified DNA complexes. Under current assay conditions and formats, we are able to detect fewer than 10 target molecules. In this paper, we demonstrated the detection of EBV using this technology.

Introduction

Ramification amplification (RAM) is an isothermal DNA amplification technology recently invented in our laboratory (1). The RAM assay utilizes a C-probe for target detection and a DNA polymerase with a high processivity and displacement activity for amplification. The C-probe contains three regions: two target complementary sequences at the 5' and 3' termini (25 nucleotides each) and an interposed generic linker region (71 nucleotides) with two primer-binding sites (Fig. 1). Once the C-probe binds to the target, the 5' and 3' regions are brought into juxtaposition, and the ends can be linked by incubation with a DNA ligase (1,2). After ligation, both forward and reverse primers and exo-Bst DNA polymerase are added to the reaction. The forward primers bind to the linker region of the ligated C-probe (Fig.1) and extended by the DNA polymerase. Since the ligated C-probe is a continuous oligonucleotide sequence lacking free termini, multimeric polymers are produced via extension of the primer along the ligated C-probe and displacement of downstream strand for many rounds, analogous to the in-vivo `rolling circle' replication of bacteriophages (3). Multiple reverse primers then bind to the nascent strands. Extension from these reverse primers results in displacement of downstream-bound reverse primers with their extended sequences. Displaced ssDNAs serve as templates for further primer extension and amplification (Fig. 1).

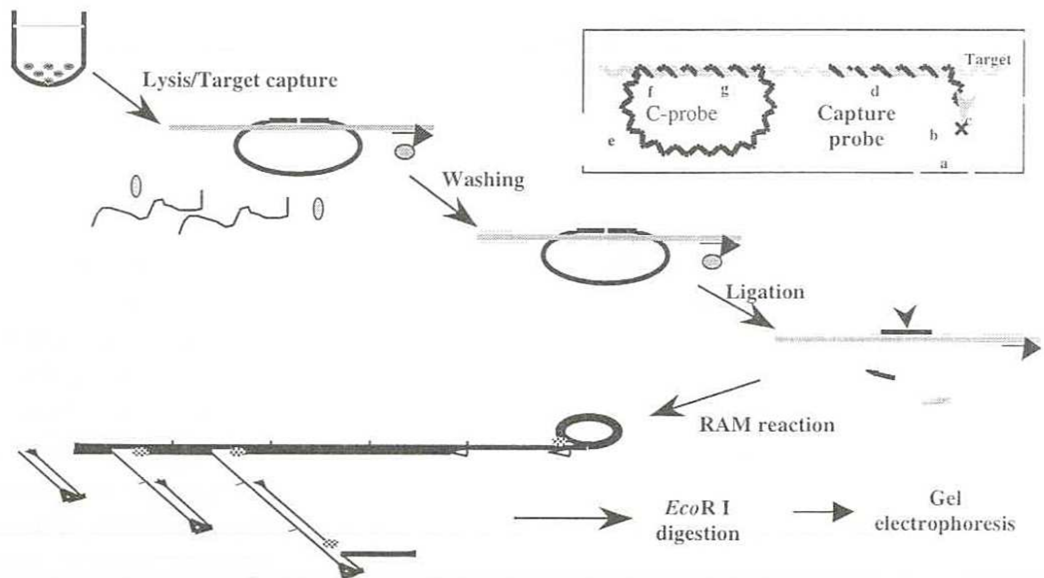

Fig 1: Schematic representation of RAM assay

Target DNA, capture probe, C-probe, and paramagnetic bead are added to hybridization buffer to allow formation of a hybrid complex. The hybrid is captured on a paramagnetic bead, allowing extensive washing to remove unbound C-probe and cellular components. The C-probe aligned on the target is linked together by a DNA ligase. RAM amplification is then carried out by the addition of forward ( ) and reverse (<) primers and DNA polymerase (●). The forward primer bound to the C-probe is extended by the polymerase and continues after one round of synthesis by displacing the bound forward primer and its extended product, generating a long ssDNA with repeated sequence.

With the reactions going on, multiple reverse primers can bind to the nascent ssDNA as their binding sites are available. Each bound reverse primer will be extended and displace the downstream primers and their extended products. The forward primer binding sites of the displaced ssDNA are then available for the forward primer to bind and extend similarly, thus forming a large ramifying DNA complex. Finally, the RAM products are examined by gel electrophoresis after EcoR I digestion. Insert: The C-probe hybridizes to the target through their complementary regions (f and g) and sequence at the noncomplementary region (e) is generic for the binding of primers. The C-probe-target hybrid is captured on a paramagnetic bead (a) through the

binding of the biotin moiety (c) of the capture probe (d) to streptavidin (b) coated on the beads.

Like the constant unfurling of streamers, multiple primer extensions are simultaneously in progress, resulting in a large ramified complex. The end-products are multimeric double-stranded DNAs (dsDNAs) of various lengths, including such smaller units as monomers, dimers, and trimers. Because the displaced DNAs are single stranded, allowing primers to bind to them at a constant temperature, the need for thermocycling to denature double-stranded DNA for the binding of the primers is obviated.

Materials and procedures

Sample preparation

Namalwa and Raji cell lines derived from human Burkitt's lymphoma were obtained from American Type Culture Collection (ATCC; Rockville, MD). These cells contain EBV and were used as positive controls. Pleural and peritoneal effusions were obtained from a patient with primary effusion lymphoma. Human leukemic cell lines, Jurkat, NB4, and U937 were obtained from ATCC and used as negative controls. All samples were lysed by incubation at 60°C for 60 min in a 300 µl solution of 5 M guanidium thiocyanate (GTC) (Sigma), 0.5 % bovine serum albumin (Sigma), 80 mM EDTA, 400 mM Tris-HCl (pH 7.5), and 0.5 % sodium-N-lauroylsarcosine (Sigma). The lysed specimens were stored at -20°C until use (4).

Rolling circle amplification with Bst DNA polymerase

10^{12} molecules of EBER targets were hybridized with 10^{12} molecules of C-probes and capture probes (5). The complex of target-C-probe-Capture probe was then captured onto magnetic beads through the binding of biotin of the capture probe to streptavidin coated on the beads. After washing and ligation, Bst DNA polymerase was added together with 1x Bst buffer containing 10^{13} molecules of forward primer and incubated at 50^{0}C for 2 hours. The products were examined on 0.8% agarose gel.

The RAM assay

Hybridization of C-probes and capture probes to targets was carried out in 80 µl reaction mixture containing 2 M GTC, 0.5% bovine serum albumin (Sigma), 80 mM EDTA, 40 mM Tris-HCl (pH 7.5), 0.5% sodium-N-lauroylsarcosine (Sigma), 50 nM phosphorylated C-probe, 2 µM capture probes (5), and 5-15 µl of lysed specimens. The reaction mixture was incubated at 55°C for 2 h to allow the complete hybridization. Then, 2 µl of magnetic beads (10mg/ml, Dynal, Lake Success, NY) in 10 mM Tris-HCl (pH 7.5), 1mM EDTA and 2 M NaCl were added and incubated to allow the beads coated with streptavidin to capture the biotin on capture probe. The beads with bound complex were then washed twice with 400 µl of TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) at room temperature to remove non-hybridized C-probes and other cellular components. Ligation is started by adding 20 mM Tris-HCl (pH 7.6) buffer containing 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD, 0.1% Triton X-100, and 12 units of Taq DNA ligase (New England Biolabs). Ligation of the 3' and 5' ends of the C-probe produces a closed circular DNA that is locked on the target. In the following step, the supernatant was removed by aspiration. RAM reaction for amplification was started by adding 20 mM Tris-HCl buffer (pH 8.8) (in 50 µl) containing 300 µM dNTP, 10 mM KCl, 10 mMM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 1.2 µM each of forward primer and reverse primer (5), 6.4 units of exo-Bst DNA polymerase large fragment (New England Biolabs), 4 ng T_4 Gene 32 protein (USB Biochemicals), and 6% dimethyl sulfoxide (DMSO) was added to the hybridize . This mixture was then incubated at 63°C for 1h for ramification reaction, followed by heating at 95 °C for 5 min to inactivate exo-Bst DNA polymerase. The RAM products (15 µl) were transferred to 5 µl mixture containing 50 mM Tris-HCl (pH 7), 15 units of EcoRI (Boehringer Mannheim), 100 mM $MgCl_2$, and 1 mM dithioerythritol and the reaction was incubated

at 37°C for 3h. Then, 15 μl of the digested products was analyzed on a 2% agarose gel.

Results

Rolling circle primer extension with Bst DNA polymerase

The result of Bst DNA polymerase for rolling circle primer extension is showed in Fig. 2.

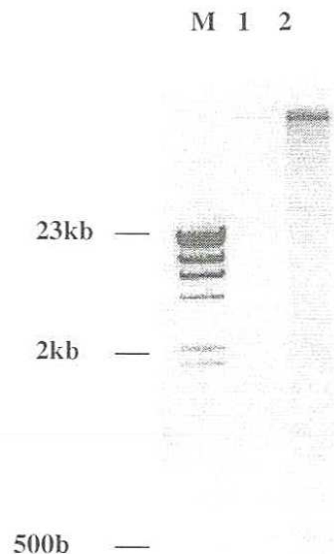

Fig 2: Rolling circle primer extension by *Bst* DNA polymerase

The result shows Bst DNA polymerase generated a large ssDNA that cannot migrate into the gel (lane 2). Lane 1: Rolling circle primer extension in the absence of target. M: DNA size marker (Lamda DNA digested with BstE II).

Bst DNA polymerase generated ssDNA much longer than 23,000 nucleotides that could not even run into the gel (Fig. 2, lane 2). The result indicates that *Bst* DNA polymerase has both a high processivity and a strong displace activity. In addition, in the absence of target, no extension products are generated (Fig. 2, lane 1), indicating the specific amplification of the C-probe.

RAM Reaction

A synthetic DNA of encoding Epstein-Barr virus early RNA-1 (EBER-1) (5) was diluted in 100-fold serials from 10^7 to 10^1 molecules and used as a target for RAM assay. Our results show that the lowest number of targets detected by this assay was 10 molecules (Fig. 3A), and the reactions were confirmed by finding the correct products (121 bp) in each lane after digestion with *EcoRI*.

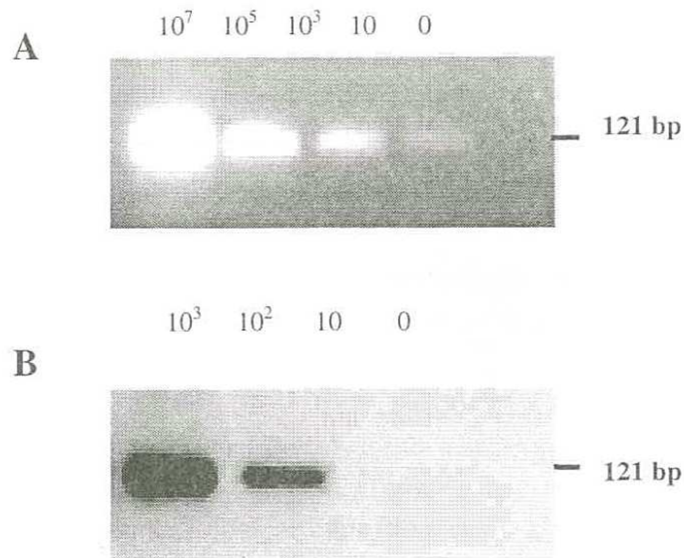

Fig 3: Detection of EBV by RAM assay

(A) *The RAM reactions were initiated with the synthetic EBER targets from 107 to 10 copies. The results shown that 10 copies of EBER targets were detected. (B) Cultured Raji cells that are stably infected with EBV were diluted from 1000 to 10 cells. As few as 10 cells were detected.*

This result indicated that the C-probe is the source of the RAM product. In the absence of target molecule, no DNA was produced, proving the target-dependent amplification of the C-probe. To further determine the sensitivity of the RAM assay, Raji cells infected with EBV were tested. Raji cells were diluted ten-fold from 1000 to 10 cells and used in RAM assay. The results show that EBV from as few as 10 Raji cells was detected (Fig. 3B).

Discussion

RAM represents a novel amplification strategy different from PCR. PCR amplifies the template DNA using a mechanism analogous to that of *in vivo* DNA replication (6). However, alteration of reaction temperatures is required for denaturation of dsDNA, annealing of PCR primers to each template, and extension of bound primers by DNA polymerase. The DNA sequence flanked by primers is duplicated in a semi-conservative manner in each cycle, generating a defined length of DNA fragments. Repeating the cycles results in an exponential amplification of the template DNA.

RAM was designed to utilize a complete, non-natural mechanism to amplify DNA sequence. A ligated C-probe serves as a template and exponential amplification is achieved through primer extension, displacement of downstream sequences, and formation of ramifying complex (Fig. 1). The reaction can be expressed as 2^u, where U is number of repeats generated from the closed C-probe. Since the displacement of the downstream strand by DNA polymerase generates ssDNA which can be bound by primers, the need for denaturation of dsDNA is eliminated.

The RAM assay offers several advantages over other amplification techniques: (1) the primers readily bind to ssDNAs displaced by the DNA polymerase, enabling the reactions to be performed under isothermal conditions, obviating the need for a thermocycler; (2) universal primers are used to amplify all probes with equal

efficiency, resulting in better multiplex capability than conventional PCR (1,7); (3) both ends of the probe can be ligated regardless of the nature of target (DNA or RNA), eliminating the need for reverse transcription for detecting RNA and creating a uniform assay format for both RNA and DNA detection(7); and (4) ligation requires that both probe termini hybridize with perfect matching, permitting the detection of a single-nucleotide polymorphism.

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VACCINATION AGAINST HERPES SIMPLEX VIRUS - WILL NOVEL APPROACHES OVERCOME PAST FAILURES?

Rouse BT, Gierynska M, Toka F, Kumaraguru U
Department of Microbiology
The University of Tennessee
Knoxville, TN ,USA

Abstract

Herpes Simplex virus infection remains a highly prevalent disease-causing agent in all societies. To date, despite multiple efforts, satisfactory vaccines do not exist. The infection is vaccine refractory for several reasons. Best understood are latency and a number of immune evasion maneuvers. Moreover, HSV control demands a successful therapeutic as well as potent prophylactic vaccines. The talk will briefly discuss some attributes of HSV that provide obstacles to vaccine development, provide suggestions why some exposed individuals appear to resist infection or fail to express lesions upon reactivation. Recent observations have indicated that a favorable outcome of HSV infection in humans best correlates with a good CD8+ T cell response, although, some favor a principal role for type 1 CD4 T cells. In mouse models, T cell immunity appears crucial for recovery with the CD8+ T cell component valuable for defense against virus in nervous tissue. With defective CD8+ T cell immunity, herpes encephalitis become a possibility. In the LT-/- mouse model, the CD8+ T cell functional defect can be compensated in part by the use of DNA vaccines encoding appropriate chemokines. Vaccines which engender functional long lasting CD8+ T cell immunity perhaps against target proteins or peptide are being explored. Preparations include the use of bioactive CpG ODN-containing vaccines as well as peptides linked to certain chaperones. Data will be presented to describe the potency and duration of immune responses to such vaccines. Finally, it may be necessary to administer vaccine combinations and giving them by appropriate application routes. The relevance of using the mucosal route is discussed with data showing that prime-boost immunization by this route may provide a different response pattern than prime-boost by the systemic route. The vaccination saga for HSV lacks a happy ending but the unfolding story tells a curious tale.

Introduction

Control of infectious disease with vaccines stands as the greatest achievement of medical science. Alas not all human pathogens have been successfully controlled with vaccines. Some major ones that have proven refractory include malaria, human immunodeficiency virus and tuberculosis. Less devastating yet significant agents include herpes simplex virus (HSV), viruses of the common cold, respiratory syncytial virus, rotaviruses, and many parasite infections. The focus of this article is on HSV types 1 and 2, agents that infect up to 80% of most populations causing significant periodic lesions in at least one third of seropositive individuals. For HSV, there is both a need for effective prophylactic as well as therapeutic vaccines.

Herpes is a disease known since antiquity being described, for example, by Hippocrates. The genital form of infection was first noted in the 1700s by Austruc, a physician for the King of France. Following the first isolation of virus early in the last century and the realization that infection induced neutralizing antibody, production of a successful vaccine was anticipated. The first candidate appeared in 1921, this to be followed by 30-40 additional efforts since that time. Typically, vaccines seem to

work favorably upon initial testing especially when evaluated by their developers. However, none to date has proven satisfactory when tested independently by double blind testing. The latest candidate to appear was developed by Smith-Kline Glaxo. It uses a major glycoprotein of HSV (gD2) that is administered with two adjuvants (MPL and alum) (1). The vaccine was shown in a recent trial to be partially effective but only in seronegative women. Modest or unapparent effects were noted in seropositive women but vaccination provided no benefit to men. Hence, this vaccine produced at enormous cost also appears far from satisfactory even for prophylactic use. The major market for HSV vaccines is for therapeutic use, which represents an even more difficult to achieve objective.

Since HSV infection invariably results in a notable humoral and T cell mediated immune response (2), why vaccines fare so poorly becomes a relevant question. No singular answer is at hand, but the principal explanation lies with the fact that the virus establishes an alternative virus host cell relationship termed latency. Latency occurs in nerve cell bodies soon after infection and remains a permanent state. In spite of intense study, the mechanism by which latency is established, maintained and eventually discontinued in some cells remains mysterious. During latency, HSV gene expression is totally different from that occurring during productive infection. Most agree that the virus only expresses a latency specific transcript called LAT that is not translated (3). Thus during latency, infected neurons appear not to express viral proteins that could signal the immune system to recognize and destroy them. Some workers contend that some viral proteins of productive cycle are in fact made and that the immune system, most particularly the CD8+ T cell immune mechanisms, is involved in maintaining latency (4). Should this prove true, then conceivably vaccines could be designed that will contain latency indefinitely avoiding the periodic termination that can result in clinical reactivation and viral spread.

Latency is not the only strategy the virus employs that appears to blunt the protective efficacy of the immune system. Others include an ability to impair antigen processing by dendritic cells (5). This could be important following viral reactivation from latency since it could result in a delay in generating effector T cells from their quiescent memory precursors. Consequently, a wider window of opportunity is available for virus to replicate in and destroy cells prior to the recruitment of a protective effector T cell response. Other blunting effects of HSV on the immune system have also been described. A list of such effects appears in Table 1.

Table 1: Some Immune Evasion Strategies of HSV

- Blocking of antigen processing by interfering with the function of the peptide transporter molecule (TAP)
- Down regulation of immune guidance molecules such as MHC
- Inhibition of complement mediated cell lysis
- Induction of fratricide between killer cells
- Inhibition of signalling pathways in dendritic cell involved in immune induction
- Encoding Fc receptors that disrupt correct antibody binding

Burning Issues in HSV Pathogenesis

As mentioned above, HSV infects up to 80% of the population but a significant percentage resist infection and remain seronegative even though many of these are doubtless exposed to the virus. This observation is difficult to explain, but it may relate to the variable efficacy of innate defense mechanisms. Accordingly, studies in animal model infection systems have revealed that several aspects of innate immunity appear capable of curtailing infection. A list of such mechanisms appears in Table 2 and this issue has been discussed in more detail elsewhere (6).

Table 2: Innate Defense Mechanisms That Affect Susceptibility To HSV

- Natural killer cell function
- Interferons and other cytokines produced in response to infection
- Antiviral effector function of macrophages and neutrophils
- Natural antibody levels
- Complement system
- Gamma delta T cell function

We have advocated that the antiviral efficacy of innate immunity may in fact be influenced by the nature of the environment in which individuals are domicile (6). This is referred to as the hygiene hypothesis, an idea now popular to explain to rising tide of allergic syndromes in the Western world (7). As it relates to HSV susceptibility, we contend that the environment and experience with other pathogens, especially early in life, may serve to influence susceptibility to primary infection by HSV (see Table 3).

Table 3:Conditions Favoring Increased Susceptibility To HSV Infection

- Quiet innate defenses – low threshold to infection
- Cellular and cytokine environment at time of infection that favors Th2 T cell induction – more susceptible
- If pattern of memory response favors $CD4^+$ Th2 and non-cytolytic $CD8^+$ then reactivation more likely to be clinical
- Over active regulating response at time of reactivation

Thus quiet innate defenses, perhaps a consequence of domicile in a microbiologically non-challenging location, could mean that the threshold dose of virus required to cause patent infection is low. A cellular and cytokine environment that favors the induction of Th2 type T cell responses at the time of infection may also result in a less effective anti-HSV T cell response that in turn will impact on the memory T cell pattern. Hence during reactivation, the T cells recalled will also favor Th-2 which intrinsically are less effective at controlling infection. Accordingly, it could be that the type of immune response generated upon initial viral interaction impacts not only on the control of primary infection, but also influences the efficacy of recall immunity following reactivation. We contend that the "right mix" could well include type 1 cytokine producing CD4+ T cells, CD8+ T cells that are functionally cytotoxic and produce abundant IFNγ upon antigen exposure and finally the absence of impeding regulatory T cell responses (see Table 4).

Table 4: The "Right Mix" Of Immune Reactivity To Assure Resistance To HSV Reinfection Or Lesions Following Reactivation

- Type I cytokine – producing $CD4^+$ T cells, esp. IFNγ
- $CD8^+$ T cells that are functionally cytotoxic and produce abundant
- IFNγ upon antigen recognition
- Absence of impeding regulatory responses

In the majority of individuals infection does occur and latency is established. A proportion of such individuals will then experience clinical recurrences which in some cases is an all too frequent occurrence. With HSV-2 genital infection, some persons secrete infectious virus almost continuously although usually such individuals are asymptomatic (8). Once again a satisfactory immunological explanation that

accounts for the differences in lesion expression is not available. It has been hypothesized that should an individual generate an appropriate type of immune response upon primary infection then this pattern holds true during recall from memory and will effectively control virus minimizing the likelihood of clinical recurrences (6). Studies with patients that are coinfected with both HSV and HIV would seem to indicate that favorable virus-host relationship (i.e., sub or minimally clinical) may largely depend on the efficacy of the CD8+ T cell response (9). A summary of the findings of the Corey and Posavad group appear in Table 5.

Table 5: Outcome Of HSV Infection In HIV$^+$ Patients

Anti-HSV T cell response pattern	Usual Consequences of HSV reactivation
Good CD4 and CD8	Mild, infrequent lesions, minimal virus
Bad CD4 and CD8	Frequent severe lesions, high virus secretion
Bad CD4 and Good CD8	Good Control, low virus secretion
Good CD4 and Bad CD8	Poor control, high virus secretion

The frequency of HSV-specific CD8$^+$ pCTL in HSV$^+$HIV$^+$ individuals was significantly lower than in HSV$^+$HIV$^-$ individuals and was not different than in HSV$^-$HIV$^-$ individuals. HIV$^+$ patients who suffered more severe genital herpes recurrences had significantly lower HSV-specific CD8$^+$ pCTL frequencies than those patients with mild recurrences. In contrast, no significant difference was seen in proliferative precursor frequencies between those patients with mild vs. severe genital herpes. Quantitative differences in pCTL frequency to HSV appear to be the most impòrtant host factor influencing the frequency and severity of HSV reactivation in HIV$^+$ patients.– Posavad et al(9)

Such observations indicate that when designing vaccines, particularly for therapeutic use, we should pay particular attention to the efficacy with which such vaccines induce the right mix of immune reactivity (see Table 4), in particular the CD8+ T cell response.
Most experimental HSV vaccine studies are performed using mouse model systems. This is far from a perfect situation especially since mouse models tend to exaggerate the efficacy of vaccine candidates. In mouse models, CD8+ T cell mediated immunity was shown to be particularly important for clearing virus from nervous tissue and perhaps from mucosal tissue (2, 10). An issue becomes how do we optimally induce CD8+ T cell responses with vaccines. The principal points relating to this issue are listed in Table 6.

Table 6: Turning On Antiviral CD8+ T Cells In Humans

- Replication deficient HSV mutants - mixed track record
- Replicating vectors eg. Vaccinia – Unpopular
- DNA vaccines – Poor record in primates unless part of a combination approach
- Exploitation of cross-priming with adjuvants given with proteins or peptides - untested
- Chaperones bound to peptides - untested

Our laboratory has recently focused on vaccines that successfully induce CD8+ T cell immunity particularly at the mucosal location. As has been shown by many groups, DNA vaccines represents one convenient and usually effective means of inducing a CD8+ T cell response (11). However, used alone, even if administered repeatedly, they have suboptimal immunogenicity even in mice and especially if used mucosally (12). In primates, immunogenicity appears to be even less. One way around the suboptimal performance was to exploit a prime-boost immunization format (12). In this format, superior immune responses were achieved if DNA vaccines were used to systemically prime animals followed by boosting with an alternative type of vaccine. For the latter, several different vaccines have been used. These include recombinant poxviruses, alphaviruses, and adenoviruses (13). Since HSV infection frequently occurs by way of the mucosal route, our focus was on the extent of immune induction at this site. Accordingly, experiments were done to compare the efficacy of anti-HSV immunity at both systemic and mucosal sites following immunization either mucosally, systemically, or a combination of the two routes (14). The results of a rather complex series of experiments are summarized in Table 7, and are described in detail in reference 14.

Table 7: Prime Boost Immunization Via Mucosal Route Changes The Rules

	gB Antigen		Response[3]	
	Prime	Boost	Systemic	Mucosal
Mucosal	DNA[1]	DNA	+	±
	DNA	VAC[2]	++	++
	VAC	DNA	++++	++++
	VAC	VAC	++	++
Systemic	DNA	DNA	++	±
	DNA	VAC	+++	±
	VAC	DNA	++	±
	VAC	VAC	+	±

[1]gB DNA
[2]Recombinant vaccinia virus encoding gB
[3]Data described in detail in reference 14

Basically these results confirm that optimal systemic immune responses can be induced if a DNA vaccine encoding the gB protein of HSV is used to prime animals and a recombinant vaccinia virus encoding gB is employed for boosting. A different set of rules hold true when mucosal immunity was analyzed. To induce optimal mucosal immunity, we found that best results came when DNA vaccines were used to boost animals that had already been primed mucosally with the recombinant vaccinia virus vector (14). Systemic boosting with DNA vaccine also induced excellent mucosal immunity provided the animals had been mucosally primed with recVACgB. Since the mucosal prime-boost format also induced excellent systemic immunity, mucosal immunization represents a valuable alternative means of vaccination to achieve a more global state of immunity. It is to be emphasized, that the mucosal immune state included surfaces remote from the site of mucosal immunization. Thus in such studies, vaccines were given intranasally, but immune efficacy was tested in the vaginal and gut mucosa (14, 15).
The above mucosal prime-boost experiments were evaluated in terms of the CD4+ Th1 T cell and antibody responses induced. As mentioned previously, a more critical parameter may be the CD8+ T cell response, particularly for protection against infection of nervous and mucosal tissues. More recently we have studied the efficacy of vaccination approaches that exclusively induce CD8+ T cell responses that react with HSV. One of these uses bioactive CpG oligodeoxynucleotides given as an adjuvant along with a small peptide that represents the predominant CD8+ T cell epitope recognized by the C57BL6 mouse strains following infection with HSV (16). Our studies show that systemic immunization with CpG-peptide results in a CTL response comparable to that induced by virus or recombinant vaccinia viral vectors that express the peptide (Table 8).

Table 8: Immunization With Cpg ODN Linked To A $CD8^+$ Peptide Epitope

Immunogen	Acute phase [a]			Memory phase [a]		
	Response[b]		Survival (%)[c]	Response		Survival (%)
	% Tet	%CTL (100:1)		% Tet	%CTL (100:1)	
gB protein	nd[d]	<5	nd	nd	nd	nd
gB + bioactive CpG	nd	42	nd	nd	nd	nd
gB + control CpG	nd	<5	nd	nd	nd	nd
Peptide	0.1	<5	0	0.1	<5	0
Peptide + bioactive CpG	6	47	92	1.1	19	54
Peptide + control CpG	0.4	<5	0	0.2	<5	0
Rec Vac-peptide	13.8	66	100	3.9	46	61
Buffer	0.1	<5	8	0.1	<5	0

[a]Acute phase mice were sacrificed on day 7 (acute) and day 60 (memory) after second immunization and analyzed for tetramer positive $CD8^+$ T cells, CTL activity by ^{51}Cr release assay or vaginally challenged with HSV and survival rate was established. The tetramer values are the pecentage of double-positive cells ($CD8^+/Tet^+$). The CTL data are expressed as a percentage of killing at effector:target (E:T) ratio 100:1.
[b]Response measured by antigen specific tetramer staining and ^{51}Cr release assay
[c]Survival rate- mice were intravaginally challenged with HSV during acute phase or memory phase post second immunization and survival rate was established
[d]nd- not done

Moreover, in the acute phase CpG peptide immunized animals, which had only a peptide-specific CD8+ T cell response, were resistant to challenge via the vaginal mucosa. The CpG-peptide immunization approach was poorly immunogenic when used mucosally in naive animals. However, if CpG-peptide was given intranasally to mice mucosally primed with RecVAC peptide, then a prompt and effective mucosal immune response did occur. As currently used, the CpG-peptide immunization approach represents a useful means of inducing CD8+ T cell mediated immunity in the acute phase, but it provides only a modest memory response. We are currently investigating if CpG-peptide immunization given systemically or mucosally could represent a valuable way to selectively boost CD8+ T cell immunity in animals long after primary infection. A similar approach could prove useful for human use.
The second immunization protocol under evaluation as a means of selectively inducing CD8+ T cell immunity uses an homologous heat shock protein to chaperone peptide epitopes (17). Thus when used for systemic immunization. Hsp70 linked to the SSIEFARL peptide induces an excellent primary CD8+ T cell response. Animals are well protected against systemic challenge in the acute phase but the durability of immunity was less than could be induced either by UV inactivated virus or RecVAC-peptide immunization (Table 9).

Table 9: Comparison of Immunity following Immunization with different preparations[a]

Immunogen	Phase[b]	Frequency(%)			CTL (LU)		
		Tetramer	ICG	ICG/Tet %[c]	Peptide	HSV	HSV/Pep%[c]
rhsp70-SSIEFARL	Acute	7.8	6.9	88	72	64	89
	Memory	2.1	1.1	52	15	4	27
*UV*HSV	Acute	6.0	5.8	97	53	50	94
	Memory	3.2	2.9	91	36	32	89
VvgB	Acute	8.1	7.3	90	82	72	87
	Memory	2.6	1.8	69	32	22	68

[a]The mice were sacrificed on day 7 (acute) and day 90 (memory) after the second immunization and analyzed for tetramer-positive CD8 T-cells, peptide-induced IFN-production, and CTL activity by 51Cr release assay as described in Materials and Methods. The tetramer and ICG values are the percentage of double-positive cells (CD8+/Tet+ and CD8+/IFN-+, respectively). The CTL data are expressed as lytic units (LU) and show results accrued from only the UV-treated HSV, VvgB-, and rhsp70-peptide-immunized groups for SSIEFARL-pulsed and HSV-infected targets.
[b]Acute phase, 7 days after the second immunization; memory phase, 90 days after the second immunization.
[c]ICG/Tet%, (intracellular IFN positive cells/tetramer-positive cells) x 100; HSV/Pep%, (HSV targets/peptide targets) x 100.

The reason for the poor memory response appeared to be explained in part by the lack of helper-cell stimulation at the time of initial immunization. Thus immunization with vaccines that solely induce CD8+ T cell responses may not be adequate to achieve satisfactory immunity. They may, however, prove highly useful to expand functional CD8+ T cell immunity in already antigen primed individuals. This issue is currently under evaluation. Ways are also being sought to facilitate the efficiency of CD8+ T cell immune induction by increasing the cellular interaction between naive responder antigen peptide specific T cells and antigen presenting cells. Accordingly, our studies have shown that LT-/- mice generate poor CD8+ T cell responses upon infection with HSV (18). The response that is made tends to be functionally defective and animals are prone to develop herpes encephalitis upon HSV challenge. We have found, however, if such mice are pretreated with the CCR7 ligand chemokines, that facilitates interaction between naive CD8+ T cells and APC, superior CD8+ T cells responses were induced. Such treated animals were protected from encephalitis (19) (See Fig. 1).

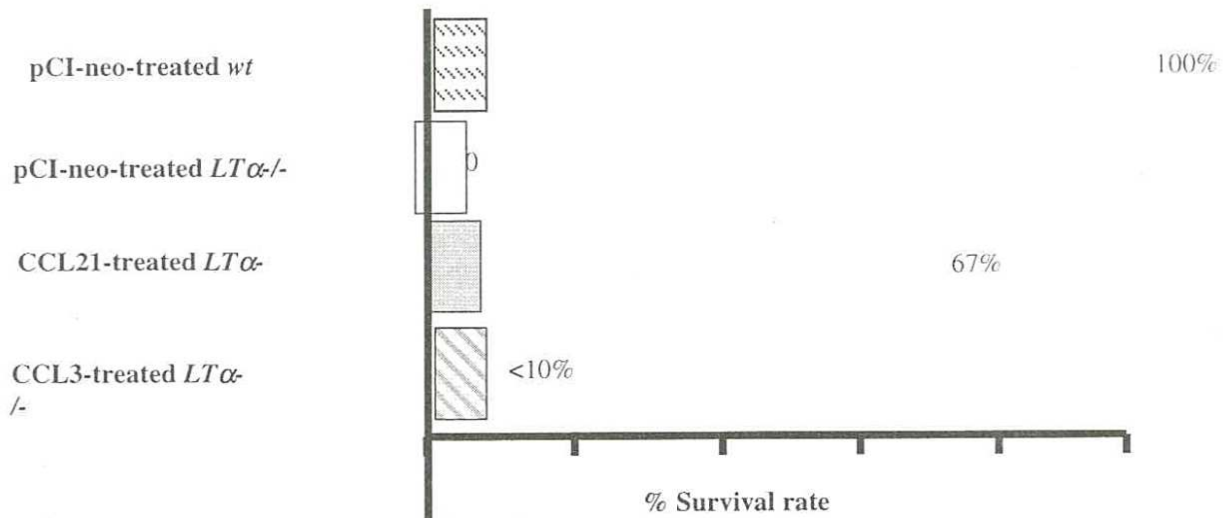

Figure 1: CCR7 ligand administration boosts immunity to HSV challenge

Five- to 6-wk-old wt and LT-/- mice previously given control vector pCI-neo or CCR7 ligands were challenged i.m. with a lethal dose (5 LD50, 107 PFU) of HSV-1 KOS. They were examined daily starting from day 3 for signs of HSV infection that include mobility, wasting, limb paralysis, and encephalitis. The figure shows survival rate on day 10 postchallenge.

Coadministration of chemokines and cytokines at the time of antigen administration promises to be a useful means of enhancing and shaping the nature of immunity. Optimistically, this and other novel approaches will result in vaccines that successful for protection against HSV. Boosting CD8+ T cell immunity could represent the therapeutic vaccine approach needed to maintain a patients asymptomatic relationship with their virus.

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MOLECULAR BIOLOGY OF HEPATITIS B VIRUS AND ITS DIAGNOSIS BY CONVENTIONAL TESTS

Ustaçelebi Ş
Hacettepe University, School of Medicine, Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

Hepatitis B virus (HBV) is a causative agent of the disease known as hepatitis B and its estimated that 400 million people throughout the world infected with HBV. This property making HBV one of the most common human patogens. The correlation between HBV and the development of hepatocellular carcinoma (HCC) has been well established. Despite to extensive research on virus replication and transformation in hepatocytes, more studies are needed before we fully understand host-virus interaction and pathogenesis.

The analysis of molecular biology of hepatitis B virus important in terms of understanding the genes coding viral proteins and analysing their functions. A great deal of studies are carried out to reveal molecular biology of HBV and this speech will be the sum of current research.

The Hepatitis B virus genome

Electron microscobic observations indicate the genome is circular, yet only partially double-stranded. HBV genome is approximately 3200 nucleotides in length. Genome basepairs are numbered according to the restriction enzyme EcoR1 or at homologous sites, if the EcoR1 site is absent. Other methods of numbering are also used, for example; the strart codon of the core protein or the first base of the RNA genome.

There are four well characterised and overlapping open reading frames (ORFs) in the genome which regulate the transcription and translation of the seven different hepatitis B proteins through use of varying in-frame start codons. For example, the small hepatitis B surface protein is generated when a translation begins at the ATG at position 155 of the HBV genome. The middle hepatitis B surface protein is generated when translation begins at an upstream ATG at position 3211. OR FP occupies the majority of the genome and encodes for the hepatitis B polymerase protein. OR FS encodes the three surface proteins. OR FC encodes both the hepatitis e and core protein.. OR FX encodes the hepatitis BX protein.

Promoters and signal regions of HBV genome

HBV genome contains many important promoter and signal regions functioning in viral replication.The four ORFs transcription are controlled by four promoter elements (pre SI, pre S2, core and X), and two enhancer elements (Enh I and Enh II). All HBV transcripts share a common adenylatian signal located in the region spanning 1916-1921 in the HBV genome.

The core/precogenome promoter controls the transcription of multiple RNAs which have 5' heterogeneity. These include the core antigen, e antigen, polymerase, and pg RNA. Viral enhancer elements and a negative regulatory element (NRE) also regulate these transcripts. The polyadenylation site is a hexanucleotide sequence

(TATAAA). Transcription factors such as OCT-1, HNF-1 and HNF-3 appear to be involved in the activation of the pre S1 promoter.

ORF X which encodes a 17 kd protein has its own promoter controlling the transcription of a 0,9 kb RNA. Both enhancer I and enhancer II can activate heterologous promoters despite their position or orientation.The properties of viral enhancers of HBV is given in Table I.

Table 1: Viral Enhancers Of HBV

Enhancer Location	Cellular Enhancer binding proteins	Enhancer properties and functions.
Enhancer I adjacent to X gene promoter	Nf-kb,Ap-1,Nf-1,bZip protein,C/ebp hepatocyte nuclear factor 3(Hnf 3) Hnf 4	Strong specificity for hepatic cells because C/ebp, Hnf 3 and Hnf 4 are sepcific for or enriched in these cells; some activity in non hepatic cells; stimulates transcription from all viral promoters in infected cells; activity may be increased by the viral X protein.
Enhancer II, adjacent to pregenomic RNA promoter.	Hnf1, Hnf3, nuclear superfamily members.	Hepatic cell specific stimulates transcription from pregenomic and pre-S promoters in infected cells.

Replication of the HBV Genome

Multiplication of the HBV genome occurs within the nucleus of an infected cell. RNA polymerase II transcribes the circular HBV DNA into greater-than-full length mRNA. Since the mRNA is longer than the actual cccDNA, redundant ends are formed. Once produced, the genomic RNA exits the nucleus and enters the cytoplasm whereupon it can be translated to generate the HBV polymerase, core and e proteins.The pregenomic RNA packaging into core particles is triggered by the binding of the HBV polymerase to the 5' epsilon stem-loop. RNA encapsidation is believed to occur as soon as binding occurs. The HBV polymerase also appears to require associated core protein in order to function. The HBV polymerase initiates reverse transcription from the 5' epsilon stem-loop three to four base pairs, at which point, the polymerase and attached nascent DNA are transferred to the 3' copy of the DR1 region. Once there, the (-) DNA is extended by the HBV polymerase while the RNA template is degraded by the HBV polymerase RNAse H activity. When the HBV polymerase reaches the 5' end, a small stretch of RNA is left undigested by the RNAse H activity. This segment of RNA is comprised of a small sequence just upstream, including the DR1 region. The RNA oligomer is then translocated and annealed to the DR2 region at the 5' end of the (-) DNA. It is used as a primer for the (+)DNA synthesis which is also generated by the HBV polymerase. It appears that the reverse transcription as well as plus strand synthesis may occur in the completed

core particle. Because of this, the finite amount of free nucleotides within the core particle may be the reason that the (+) DNA strand of the HBV genome varies in length in completed virions.

Hepatitis B surface proteins

Within the HBV genome, the region encoding the HBV surface proteins contains three in-frame start sites which share a common termination codon. Therefore, the various HBV surface proteins are all related to each other by a shared regilon known as the S-domain.

Small hepatitis B surface antigen (HBsAg or SHBsAg) is the smallest of the hepatitis B virus surface proteins, containing solely the S-domain. SHBsAg is highly hydrophobic which containing four-transmembrane regions. The HbsAg contains a high number of cysteines, each of which is cross-linked to one another SHBsAg appears to be manufactured by the virus in high quantities. It also contains a highly antigenic epitope. Analysis of this epitope allows for the subtyping of HBV carriers.

Middle hepatitis B surface antigen (MHBsAg) is intermediate or middle-sized HBV surface protein contains an additional 55 amino-acid domain known as Pre-S2. This domain is hydrophilic and reside extracellularly.

According to some evidence, this protein is involved in HBV attachment and entry into the hepatocytes. However, studies carried out with mutants indicate that pre-S2 is not required for HBV infectivity, nor viral particle morphogenesis. Large hepatitis B surface antigen (LHBsAg) is the largest of the HBV surface proteins, containing the pre-SI domain as well as the pre-S2 and S domains. The pre-SI domains's sequence appears to be highly variable, suggesting that this may be the HBV protein involved in liver attachment. The pre-SI domain contains no additional glycosylation sites, but contains a myristylation signal at the N-terminus anchoring the N-terminus to the membrane.

There are two different folding patterns of LHBsAg. The first type is found on the cell surface and in mature virions. The other is found on the surface of the endoplasmic reticulum. It appears that both pre-SI and pre-S2 domains remain cytoplasmic when the LHBsAg in the endoplasmic reticulum. The pre-S2 domain remains unglycosylated where is the pre-SI domains become myristylated. However, when the pre-S2 domains are translocated across the membrane are still unclear.

Overexpression of the LHBsAg results in endoplasmic reticulum retention of the protein LHBsAg. This protein is believed by most to be the one responsible for mediating viral attachment onto its host cells. However, the receptor for HBV has not been fully isolated yet.

Hepatitis B core " c " and "e" proteins

The core ORF of HBV encodes two sequence related but functionally distinct proteins. The hepatitis B core protein and the hepatitis B "e" protein.

The hepatitis B core protein (HBc) is the major component of the nucleocapsid shell packing the HBV genome. This protein contains 185 amino acid and expressed in the infected cell cytoplasm. HBc protein is hydrophilic and non-glycosylated. HBc is largely a helical protein with five alpha-helices. The C-terminal end of the protein contains four arginine clusters which appear to be involved in nucleotide packaging. HBc can also be phosphorylated on serin residues at position 157,164 and 172. Unlike the core protein, the hepatitis B "e" antigen has different properties. HBcAg is named "e" due to its appearance "early" in acute infection. HBe is expressed by the translation of sequences upstream of the HBc ORF known as the pre-C sequence.

The pre-C sequence encodes a hydrophobic transmembrane domain, resulting in the translation and translocation of HBe into the lumen of endoplasmic reticulum. The fundamental change in the lacation of protein expression alters the antigenicity of HBe such that it does not share antigenic homology with HBc, despite having nearly identical aminoacid sequences.In the endoplasmic reticulum, 19 of 29 residues of the pre-C region are removed by a signal peptidase. The remaining pre-C residues prevent HBe from forming into core particles. Another region in the arginine rich domain is cleaved from HBe in the Golgi complex.
The prupose of producing this secretory form of HBc is not fully understood. Synthesis of high levels of HBe may somehow suppress the immune system from eliminating HBV producing cells since high level of HBe are often found in the serum of HBV carriers.

Hepatitis B virus Polymerase protein

The largest ORF in the HBV genome encodes for the hepatitis B polymerase protein (HBp). HBp has RNA and DNA dependent polymerase activity. HBp contains four characterized domains. The N-terminus portion of the protein acts in priming (-) DNA strand synthesis and ends up covalently linked to the 5' end of the (-) DNA strand. This domain is called, also termed primase. The following domain does not seem to have any enzymatic function, but acts as a spacer between the first and third domains. The third domain is HBp portion and occupies almost 40% of the total protein. The product of this domain has RNA and DNA dependent polymerase activity. The fourth domain of HBp possesses its RNase H activity. This domain plays a key role in HBV genome replication.

Hepatitis BX protein

The smallest ORF in the HBV genome encodes for the hepatitis BX protein (HBX). It is named X because entire function and cellular localization is not fully understood HBx, consist of 154 aminoacid and has conserved sequences. All hepadnaviruses have HBx except avian hepadnaviruses.
HBx has not been found in mature virions nor associated with nucleocapsid particles. HBx is possibly localized in the cytoplasm according to sequence analysis and phosphorylated forms are exist. Some suggest that HBx has serine/threonine kinase activity. It has also been proposed that HBx upregulate the transcription of many endogenous host genes. HBx is thought to enhance the DNA binding ability of other transcription factors through interaction with their leucine zipper regions (bZip).
Perhaps the most exciting effect of HBx may be its tumorogenic activity on HBV infected cells. Overexpression of HBx in hepatocyte culture and transgenic mice results in a tumorigenic phenotype. Experimental evidences suggest that HBx interacts with the ras-raf-MAP kinase pathway to promote cell growth. Moreover, HBx has been shown to bind onto p53,a prominent cell tumour-suppressor protein.

Conventional tests for Hepatitis B virus diagnosis

A number of serologic tecniques with different degrees of sensitivity and specificity have been developed for the detection of HBV antigens and their specific antibodies (Table 2).

Table 2: Assay markers for HBV

HBsAg
Anti-HBs
Anti-HBc
IgM Anti-HBc
HBeAg/Anti HBe

Several conventional test precedures are used in the past for detection of HBV markers. (Table 3).

Table 3: Conventional tests used for HBV markers

Agarose gel diffusion
Discontinuous counterimmun electrophoresis (DCIE)
Reophoresis
Complement Fixationtest
Reverse passive latex agglutination
Reverse passive hemagglutination
Enzyme Immunassay (EI)
RadioImmunoassay (RIA)

Despite the availability of different serological procedures, EIA is the most common routine diagnostic test to detect HBV antigens and antibodies. Although EIA has replaced RIA as the most popular method for detecting the various hepatitis B assays is given in table 4.

Table 4 : Principles of procedures used in various hepatitis B assays

Serologic assay	**Principle of procedure (RIA or EIA)**	**Typeof support system**	**Adsorbed reagent**	**Label or conjugate**
HBsAg	Sandwich	Bead,well	Anti-HBs	Anti-HBs
HBeAg	Sandwich	Bead,well	Anti-HBe	Anti-HBe
Anti-HBs	Sandwich	Bead,well	HBsAg	HBsAg.
IgM anti-HBc	Sandwich (modified)[a]	Bead,well	Anti-IgM	Anti-HBc
Anti-HBc	Competitive binding[b]	Bead,well	HBcAg	Anti-HBc
Anti-HBe	Competitive binding[c]	Bead,well	Anti-HBe	Anti-HBe

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MOLECULAR DIAGNOSIS OF HEPATITIS VIRUSES
HEPATİT VİRUS İNFEKSİYONLARININ MOLEKÜLER TANISI

Badur S
İstanbul Üniversitesi, İstanbul Tıp Fakültesi, Mikrobiyoloji ve Klinik Mikrobiyoloji Anabilim Dalı, Viroloji ve Temel İmmünoloji Bilim Dalı, Çapa İstanbul, TÜRKİYE

Moleküler biyoloji alanında kaydedilen gelişmelerden tüm bilim dallarının yanı sıra elbette infeksiyon hastalıkları konusu da payını almış; bir yandan yeni tanı olanakları gündeme gelirken, profilaksi açısından da bir çok yenilik kullanıma girmiştir; örneğin bugün için dünyada yaygın olarak kullanılan hepatit B aşısının rekombinan DNA teknolojisi uyarınca, maya ya da memeli hücrelerinde üretildiğini biliyoruz. Burada , söz konusu tekniklerin tanı açısından ne tür olanaklar sağladığını ayrıntılı olarak ele alacağız; bu tip bir incelemede, moleküler biyolojinin viral hepatit etkenleri konusunda getirdiği yenilikleri iki grupda ele almanın doğru olduğuna inanmaktayım. Önce, bilinen klasik etkenlerin (örneğin hepatit A ve B virus infeksiyonları gibi) neden olduğu infeksiyonların, serolojik tekniklerle bu güne dek başarıyla gerçekleştirilen tanısının, moleküler biyoloji yöntemleri ile nasıl ayrı bir boyut kazandığı; sonuçta klasik olarak kabul edilen doğruların, yeni teknoloji ile ne oranda değişime uğradığı irdelenecektir. İkinci aşamada ise, hepatit C virusundan (HCV) başlayarak, yeni virusların belirlenmesi, tanısı veya tedavi etkinliğinin ölçümünde yine moleküler biyoloji tekniklerinin ne tür olanaklar sağladığı ele alınacaktır. Bilindiği gibi bu yöntemler ile, 1980'li yıllarda nonA-nonB hepatitleri olarak tanımlanan klinik tablodan sorumlu HCV ve hepatit E virusu (HEV) başta olmak üzere, hepatit G virusu (HGV), TT -virus (TTV) ve nihayet SEN-V ile bunların varyantları ortaya konmuş ; ve yeni tanımlanan bu virusların viral hepatit etkenleri listesindeki yerleri belirlenmiştir.
Viral hepatitlerin tanısında kullanılan serolojik ve immünokimyasal yöntemlere oranla, etkenin nükleik asidini (DNA veya RNA) saptamaya yönelik moleküler tekniklerin bir dizi üstünlüğü bulunmaktadır. Her şeyden önce, diğer tanı olanaklarına göre çok daha yüksek özgüllüğe ve duyarlığa sahip olan moleküler yöntemler bir yandan klasik tanı yöntemleriyle çözümlenemeyen soruların yanıtlanmasını, öte yandan genotip ayırımı gibi yeni kavramların gündeme gelmesini sağlamışlardır.
Hibridizasyonun yanı sıra, özellikle hedef ürün veya sinyal amplifikasyonu gibi duyarlığı arttırıcı aşamalardan sonra gerçekleştirilen uygulamalar ile virusun eser miktardaki varlığının bile gösterilebilmesi, viral hepatitlerin tanısına farklı bir boyut kazandırmış; ayrıca kantitatif moleküler teknikler kullanılarak, tedavi indikasyonunu belirlemek, etkinliğini izlemek ve gerektiğinde tedavi protokollerini değiştirmek mümkün olmuştur. Son yıllarda, otomasyon sistemlerinin geliştirilmesi sonucunda, kısaca NAT olarak tanımlanan nükleik asit testlerinin kan bankaları gibi çok sayıda örneğin birada taranmasını gerektiren merkezlerde gündeme geldiğini biliyoruz; HCV örneğinde olduğu gibi, serokonversiyon öncesi döneme ait örneklerden kaynaklanacak bulaşmaları engellenmeyi hedef alan bu tip uygulamaların, özellikle posttransfüzyonel hepatit açısından önem taşıdığı yadsınmaz bir gerçektir.
Viral hepatit etkenleri listesinde yer alan viruslara ait moleküler biyoloji uygulamalarına göz attığımızda, konunun önemi daha iyi anlaşılacaktır.

1. Oral fekal yoldan bulaşan iki klasik etken, hepatit A virusu (HAV) ve HEV açısından, moleküler teknikler daha çok araştırma alanında uygulanmaktadır. Örneğin HAV konusunda, söz konusu tekniklerin moleküler epidemiyoloji çalışmalarında kullanıldığı;

ayrıca hücre kültürüne adapte edilen attenüe HAV suşlarının nükleotid sekanslarının incelemesinde yararlı oldukları bilinmektedir. Özellikle kültürde üreme özelliğinden ve patojeniteden sorumlu mutasyonların ortaya konması, HAV aşı çalışmalarında önemlidir (1). HEV ile ilgili olarak, özellikle dışkı ve serumda viral RNA'nın moleküler tekniklerle izlenmesi, etkenin viremi özelliğinin ve dışkıdan atılma süresinin belirlenmesi açılarından yararlıdır (2).
Ancak , moleküler biyoloji teknikleri, özellikle hepatit B virusu (HBV) ve HCV infeksiyonları açısından pratiğe yönelik önemli adımlar atılmasını sağlamıştır.

2. 1988 yılında Thiers ve ark (3), seronegatif üç olguda HBV-DNA varlığını göstermişler; bu örnekleri inoküle ettikleri şempazelerde akut infeksiyonun geliştiğini saptayarak, enderde olsa tüm serolojik göstergeleri negatif bulunan kişilerin de HBV taşıyabileceklerini ve bulaştırıcı olabileceklerini kanıtlamışlardır. Bu bulgu, özellikle HBsAg negatif kanların kullanımını takiben alıcılarda ortaya çıkan ve o güne dek açıklanamayan posttransfüzyonel hepatit B infeksiyonlarının kaynağının belirlenmesi açısından önemlidir. Benzer bir durum, yine yüzey antijeni negatif ancak Anti-HBc antikorları pozitif donör kanlarının bulaşa yol açıp açmayacaklarının incelenmesi konusunda ortaya çıkmıştır. Nitekim Yotsuyanagi ve ark (4), bu tip örneklerin % 38'inde düşük titrede de olsa HBV-DNA varlığını saptayarak, kullanımlarında dikkatli olunması gereğini vurgulamışlardır. Moleküler tekniklerin bir diğer önemli katkısı, HBeAg negatif örneklerde saptanan replikasyon varlığı ve pre-kor bölgesindeki mutasyonlara bağlı olarak ortaya çıkan Anti-HBe /HBV-DNA birlikteliğinin gösterilmesi açısından olmuştur (5).
Özellikle .kantitatif HBV-DNA araştırmaları, tedavinin etkinliğini belirlemede yararlanılan ve sonuçta tedavinin yönlendirilmesini sağlayan; ve bu açıdan pratik önemi olan uygulamalardır. Günümüzde Digene Hybrid Capture system HBV -DNA assay (Digene Corp.); Cobas-Amplicor HBV Monitor test (Roche Diagn. Syst.); Versant HBV (Bayer Corp.)-eski: b.DNA-Chiron- gibi çeşitli ticari HBV-DNA testleri kullanılarak, nükleik asit miktar tayinini gerçekleştirmek mümkündür. Ayrıca Transcription-mediated amplification-TMA (Gen Probe), TaqMan teknolojisi uyarınca gerçekleştirilen real-time PCR ve ülkemizde henüz ticari olarak bulunmayan Cross-linking assay (NAXCOR) uygulamaları ile de HBV-DNA düzeyini saptamak olasıdır. Bu yöntemlerin karşılaştırmalı çalışmalarında, ticari kitlerin yinelenebilir sonuçlar verdikleri ve duyarlıklarının tedavinin etkinliğini ölçmede uygun oldukları bildirilmiştir. Hangi testin seçilmesi gerektiği sorusuna ise, uygulamayı yapacak olan laboratuarın çalışma koşullarının belirleyici olduğu; bazı kitlerin pg/ml, bazılarının kopya/ml şeklinde sonuç verdiği unutulmamalı ve hastaların izlenmesinde aynı yöntemin kullanılmasına özen gösterilmelidir (6,7). Laboratuarımızda kullandığımız ve deneyim kazandığımız üç ticari kitin özellikleri tablo 1'de gösterilmiştir.

Tablo 1: Kantitaif HBV-DNA kitlerinin özellikleri

Ürün	Deney süresi	Test sayısı	Serum miktarı	Ölçüm sınırları (Kopya/ml)
Digene Hyb. capture	5 saat	60/kit	50 µl	700.000-560.000.000
Versant – HBV	2 gün	42/kit	10 µl	700.000-5.000.000.000
Cobas-Amplicor	2 gün	42/kit	100 µl	200-200.000

*Ölçüm değerlerinde bir standardizasyon olmadığından, bu kıyaslamayı yapmanın tartışmalı olduğu bilinmektedir.

HBV konusunda, moleküler tekniklerin bir diğer uygulama alanı, genomda meydana gelen mutasyonların izlenmesidir; örneğin fulminan hepatit patogenezinde, bu tip hastalardan izole edilen suşların genomik sekanslarında ne tip farklılıkların bulunduğu ve bu değişikliklerin kliniği nasıl etkilediği araştırılmıştır (8). Son yıllarda lamuvidin gibi bazı antivirallere direnç gelişiminin izlenmesinde, yine moleküler biyoloji tekniklerinden yararlanılmakta; bu amaçla klasik RFLP tekniğinin yanı sıra, kısaca LIPA olarak bilinen (INNO-LIPA HBV DR, Innogenetics) ticari kitlerin kullanılabilineceği belirtilmiştir (9,10). Nükleozid analogları ile yapılan çalışmalar tedaviye yanıt açısından umut verici gözükmekteyse de, kısa süreli tedaviye alınan yanıtın kalıcı olmayışı nedeniyle, interferona oranla daha uzun süreli kullanımların gerekmesi çeşitli sorunları beraberinde getirmiştir. Özellikle tedavinin uzatılması durumunda, giderek artan polimeraz geni mutasyonları, ve özellikle 552. kodondaki (YMDD bölgesi) mutasyonlar , tedaviye dirençten sorumlu tutulmaktadır. İşte bu tip mutasyonların varlığını araştırmak için, amplifikasyon işlemini takiben HBV'nun P geninin ORF bölgesindeki B ve C zincirlerindeki genetik varyasyonların saptanmasında, yukarıda belirtilen INNOLIPA kitleri ile 528.,552.,ve 555. kodonlardaki değişimleri saptamak olasıdır.
Moleküler biyoloji tekniklerinin HBV konusunda uygulanması ile ilgili olarak vereceğimiz son örnek, genotipleme çalışmalarıdır. Birazdan değineceğimiz gibi, HCVyi konu olan araştırmalarda, genotip 1b suşlarının interferona dirençli olmaları örneğinden hareketle, benzer bir durumun HBV içinde geçerli olup olmadığını araştırmak amacıyla, genotipleme tekniklerine başvurulmaktadır (11). Ayrıca, bugün için varlığı kabul edilen yedi genotip grubundan (A-G), genotip C suşlarının daha ağır karaciğer yıkımına yol açtıklarını düşündüren bulgular mevcuttur (12). Genotip saptamada, RFLP tekniği; genotiplerle ilintili prekor mutasyonlarının arandığı INNO-LIPA kitleri; yada sekanslama cihazları (Visible Genetics) ile gerçekleştirilecek tipleme işlemlerinden yararlanılabilir. İstanbul Tıp Fakültesinde gerçekleştirilen ve interferon tedavisine yanıt veren (s:10) ve vermeyen (s:10) olgulardan alınan serum örneklerinin çalışıldığı araştırmada, sekanslama sonrası gen bankasının genotip-protipleri ile kıyaslanarak tiplendirme yapılmış ve tüm suşların D genotipinden oldukları saptanmıştır.

3. Viral hepatitler alanında moleküler biyoloji açısından en önemli gelişmeler HCV konusunda gerçekleştirilmiştir. Her şeyden önce 1989 yılında ilk kez üretilemeyen yada biyolojik materyellerden saflaştırılamayan viral antijenler, rekombinan

proteinler hazırlanarak elde edilmiş ve spesifik Anti-HCV antikorlarının araştırılmasında kullanılmıştır (13). Günümüzde rutin laboratuarlarında, HCV ile ilgili olarak gerçekleştirilen tek serolojik göstergenin Anti-HCV antikorları olması nedeniyle, serokonversiyon öncesi akut HCV infeksiyonlarının belirlenmesinde;antikor varlığına rağmen vireminin bulunup bulunmadığının araştırılmasında ; ve Anti-HCV oluşturamayan immünosüprese hastalarda, HCV-RNA incelemesi büyük önem taşır, Ayrıca, kısıtlı tedavi olanaklarının etkinliğini belirlemede ve gerektiğinde tedavi protokollerinin yeniden düzenlenmesinde kantitatif HCV-RNA testlerinden yararlanılması gerekmektedir. Bu amaçla kullanımda olan ve laboratuarımızda karşılaştırma olanağı bulduğumuz ticari kitlerden Amplicor HCV Monitor 2.0 (Roche Molecular Syst.) sisteminin ölçüm sınırları: 600-500.000 IU/ml; Versant HCV-RNA 3.0 Assay- eski b.DNA chiron (Bayer Corp.) sisteminin ise ölçüm sınırları: 615-7.700.000 IU/ml olarak bildirilmektedir. Bunların dışında LCx HCV-RNA (Abbott Diagn.) ve NASBA (Organon Teknika) teknikleri ile de kantitatif HCV-RNA araştırması yapmak mümkündür (14). Özellikle 1b genotipinin tedaviye dirençli olduğunun anlaşılmasından sonra, HCV suşlarını genotipleme çalışmaları yaygın olarak kullanılmaya başlanmıştır; bu amaçla RFLP tekniğinin yanı sıra, yine INNOLIPA HCV-II (Innogenetics) yada 5'bölgesinin direkt sekanslanması esasına dayanan Trugene HCV 5'NC Genotyping (Visible Genetics) kitlerinden yararlanılabilinir (15). İstanbul Tıp Fakültesinde genotipe özgü primerlerin kullanıldığı PCR yöntemi uyarınca gerçekleştirdiğimiz ve 260 HCV suşuna ait tiplendirme çalışmasında, örneklerin %69'unun genotip 1b oldukları saptanmıştır.
HCV ile ilgili moleküler biyoloji çalışmalarına son örnek, özellikle çok sayıda muayene maddesinin tarandığı kan bankalarına yönelik Nucleic Acid Amplification Technology (NAT) uygulamasıdır . Günümüzde tüm dünyada donör taramalarında Anti-HCV araştırması yapılıyor ise de, serokonversiyon süresinin değişkenliği, antikorların bazı olgularda geç ortaya çıkması ve ender de olsa hiç oluşmaması, bu tip merkezlerde HCV –RNA taramalarını gündeme getirmiştir. Moleküler biyoloji tekniklerinin pahalı ve karmaşık uygulamalar olması nedeniyle, kısaca NAT projeleri olarak tanımlanan bu yaklaşımlarda, belirli sayıda örnekten bir havuz oluşturulması ve buradan hareketle incelemenin yapılması öngörülmektedir (16). 2000'li yıllarda bazı batı ülkelerinde rutin kullanımları başlatılan bu tip uygulamalar ile, HCV açısından pencere döneminin % 45-72 oranında azaltılabileceği ve örneğin Fransa'da tüm donasyonlar için yılda ilave 12 HCV pozitif örneğin belirleneceği hesaplanmış (17); ayrıca Anti-HCV testinde 70 gün olan pencere döneminin 41-60 gün kadar azaltılarak infeksiyon riskinin düşürüleceği öngörülmüştür (18,19) . Ülkemiz için bir model oluşturması açısından, yurtdışı destekli bir proje kapsamında İstanbul'da Çapa Kızılay Kan Merkezinden sağlanan 10.560 donör kanında bu uygulama gerçekleştirilmiş ve 27 örnekde (% 0,26) HCV-RNA saptanmıştır. Ancak retrospektif değerlendirmelerde, NAT uygulaması ile pozitif bulunan tüm örneklerin Anti-HCV açısından da pozitif bulunmuş olması, bu kısıtlı sayıda örneğe uygulanan teknolojinin ülkemiz için ilave bir getiri sağlamadığını düşünmekteyiz. (Aynı uygulamanın, HBV infeksiyonlarının sık görüldüğü ülkelerde de gündeme gelmesi söz konusundur!)

4. Yeni hepatit virusları açısından moleküler biyoloji tekniklerinin büyük önem taşıdıkları bilinmektedir. Her şeyden önce HGV, TTV ve nihayet SEN-V un saptanmasında, representative differential amplification (RDA) tekniğinin kullanıldığını; ayrıca bu üç yeni virusun gerçekten hepatotrop etkenler olup

olmadıkları; veya ne oranda patojen oldukları sorularına yanıtların, moleküler biyoloji teknolojisi ile araştırıldığını biliyoruz. Örneğin HGV'nun tanımlanmasının yanısıra, söz konusu virusun HBV/HCV koinfeksiyonlarında karaciğer hasarını ne oranda arttırdığı; ya da HGV varlığının, bu tip olgularda tedaviye yanıtı etkileyip etkilemediği, çeşitli moleküler yöntemlerle araştırılmıştır (20,21). Benzer teknolojiler kullanılarak tanımlanan TTV 'nin, örneğin ülkemizde yapılan çalışmalarla da, kronik B ve C hepatitlerinin yanısıra, HIV pozitif olgularda, kriptojenik hepatit hastalarında, veya politransfüze olgularda ne oranda bulunduğu semi-nested PCR kullanılarak TTV-DNA araştırması ile belirlenmiştir (22). Ve nihayet viral hepatit etkenleri(!) arasında son sırayı işgal eden SEN-V 'nun 1999 yılında tanımlanmasından sonra, etkeni saptayacak primer dizilerinin açıklanması ve farklı gruplarda bu virusun araştırılması gündeme gelmiş ayrıca gerçek bir patojen olup olmadığı sorusuna yanıt aranmaya başlanmıştır (23,24).

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POSTER PRESENTATIONS

DAY 1

22.APRIL.2002

1-01

A COMPARISON OF THREE DIFFERENT MOLECULAR METHODS FOR IDENTIFICATION OF *MYCOBACTERIAE*

Saniç A[1], Eroğlu C[2], Günaydın M[1], Güdül-Havuz S[1], Bayramoğlu G[1], Akkurt L[1]
Ondokuz Mayıs University, Medical Faculty, [1]Dept.of Microbiology and Clinical Microbiology, [2]Dept.of Clinical Microbiology and Infectious Diseases, Samsun, TURKEY

In this study, PCR-RFLP, Accuprobe® and INNO-LIPA Mycobacteria® have been compared for the identification of mycobacteria which was grown in radiometric Bactec 12B bottles. The PCR-RFLP method is based on the amplification of a 439-bp segment of the 65-kDa-heat shock protein (*hsp65*) and the restriction enzyme analysis of PCR products using *Bst*EII and *Hae*III. The pattern of restriction is compared to a published algorithm (about 28 species). *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. gordonea* and *M. kansasi* can be identified through Accuprobe® kit. INNO-LIPA Mycobacteria® kit can identify *M. chelonae*, *M. xenopi*, *M. paratuberculosis*, *M. silvaticum*, and *M. scrofulaceum* in addition to above mentioned strains.

The results of identification methods for 34 mycobacteria have been well matched. 28 *M. tuberculosis* complex, 2 *M. gordonea* have been identified by three methods and found conforming each other. Both PCR-RFLP and INNO-LIPA Mycobacteria® have identified one *M. chelonae*. Two isolates have been identified as *M. fortuitum* by PCR-RFLP. One of the mycobacterium isolates has not been identified in terms of species; however, it has been identified as mycobacteria spp. by INNO-LIPA Mycobacteria® test.

These methods can be considered to be suitable methods for the routine identification of mycobacterium because they are easier and more rapid than conventional identification techniques, which identify mycobacterium according to their morphological and biochemical properties, and can provide the result within a day.

1-02

TYPING OF NONTUBERCULOUS MYCOBACTERIA BY POLYMERASE CHAIN REACTION-RESTRICTION ENZYME ANALYSIS METHOD: RESULTS OBTAINED IN HACETTEPE UNIVERSITY BETWEEN 1997-2002.

Ergin A[1], Alp A[2], Ercis S[2], Kocagöz T[2], Hasçelik G[2].
Hacettepe University, [1]School of Health Services, [2]School of Medicine, Department of Microbiology and Clinical Microbiology, Ankara, TURKEY.

Polymerase-chain reaction restriction analysis method (PRA) is a rapid, practical and safe method for typing of mycobacteria to the species level.

In this study, a total of 340 mycobacterial strains isolated from clinical specimens which gave positive growth index in the BACTEC 460 radiometric system in Hacettepe University Hospital Clinical Pathology Laboratories were evaluated by PRA between 1997- January 2002. 281 of 340 strains (82.6%) were identified as *M. tuberculosis*, and 59 (17.4%) were identified as non-tuberculous mycobacteria (NTM). Eleven (18.6%) of the NTM strains were *M.gordonae I*, 6 (10.1%) were *M.avium*, 5 of (8.5%) each were *M.gordonae IV*, *M.mucogenicum*, 4 (6.8%) of each were *M.peregrinum*, *M.lentiflavum*, 3 (5.0%) were *M.chelonae*, 2 (3.4%) of each were *M.gordonae III*, *M.phlei*, *M.fortuitum*, one of each (1.7%) were *M.flavescens*, *M.malmoense*, *M.porcinum*, *M.smegmatis*, *M.triviale*, *M.simiae*, *M.vaccae*, *M.gordonaeVII*. Seven isolates (11.9%) had PRA patterns that did not match any patterns previously described.
As a result, in this five year time, PRA is found to be a reliable method for typing of mycobacterial isolates to the species level. This important data can help to learn about the epidemiology of the mycobacterial species and selection of a proper treatment regimen for tuberculosis.

1-03

MOLECULAR EPIDEMIOLOGIC CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM İZMIR, TURKEY

Biçmen C [1], Williams-Bouyer N [2], Pan X [2], Ramaswamy S [2], Şenol G [1], Graviss E [2]

[1]Training and Research Hospital for Chest Diseases and Surgery, Microbiology Laboratory, İzmir, TURKEY

[2]Baylor College of Medicine, Department of Pathology, Houston, Texas, USA

Objectives: The aim of this study was to molecularly characterize clinical isolates of *M. tuberculosis* from the İzmir region in Turkey.

Methods: A total of 55 *M. tuberculosis* isolates recovered from patients suffering from pulmonary tuberculosis were characterized by IS*6110* RFLP and spoligotyping. These isolates were also assigned to one of three major genetic groups based on polymorphisms found in codons 95 and 463 of *gyrA* and *katG,* respectively.

Results: IS*6110* profiling identified fifty fingerprint patterns with copy numbers ranging from 2 to 21. Nine isolates were identified as low-copy number (≤4) isolates. Forty-five isolates had unique fingerprint patterns and the remaining ten isolates fell into five clusters. Of the 35 spoligotype patterns identified, five spoligotypes were associated with low-copy number isolates. In contrast to IS*6110* RFLP clustering, 29 isolates were clustered into 9 spoligotype patterns. Thirty-eight isolates belonged to major genetic group 2, 16 isolates belonged to group 3 and one isolate was identified in group 1.

Based on all three genotypic methods, five clusters with two isolates each were identified.

Conclusions: Our results indicate that IS*6110* profiling and spoligotyping together provide increased molecular discrimination of *M.tuberculosis* genotypes from the İzmir region in Turkey.

1-04

DETECTİON OF RİFAMPİCİN RESİSTANCE İN *MYCOBACTERİUM TUBERCULOSİS* ISOLATES BY A RAPİD PHAGE AMPLİFİCATİON ASSAY

Biçmen C , Şenol G, Coşkun M, Florat N
Training and Research Hospital for Chest Diseases and Surgery,
Microbiology Laboratory, Izmir , TURKEY

Objectives: It was aimed to perform rapid determination of rifampicin resistance in *Mycobacterium tuberculosis* clinical isolates by a phage amplification assay and evaluate its overall accuracy.
Methods: A total of 32 isolates of which the susceptibility patterns were analyzed by the absolute concentration and BACTEC 460 TB system were taken into the study. Seventeen of them were resistant whereas 15 were susceptible to rifampicin. Rifampicin resistance for these two groups was detected by a commercially available phage amplification assay; *FastPlaque*TB-*RIF* (Biotec Lab. Ltd., UK) according to the manufacturer 's recommendations.
Results: Fifteen of 17 rifampicin-resistant isolates were found as resistant and fourteen of 15 rifampicin-susceptible isolates were susceptible by *FastPlaque* respectively. Two resistant and one susceptible isolates were evaluated as intermediate. Sensitivity, specificity and overall accuracy were calculated as 88.2 %, 93.3 % and 90.6 % respectively.
Conclusions: *FastPlaque*TB-*RIF* can be useful as a rapid (in two days' time) and practical method for rifampicin resistance and this might allow alterations in treatment regimens in time to take tuberculosis under control.

1-05

RAPID DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN CLINICAL SAMPLES BY USING *FASTPLAQUE* TB TEST

Alp A, Hasçelik G
Hacettepe University Medical School,
Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

The reemergence of tuberculosis in conjunction with the increasing number of multi-drug resistant strains of *Mycobacterium tuberculosis* has increased the need for rapid diagnostic methods. *FASTPlaque*TB test uses specific mycobacteriophage to reflect the presence of viable *M. tuberculosis*. Zones of clearing (plaques) in the cell lawn indicate the presence of *M. tuberculosis* in the sample. The aim of this study was to compare BACTEC460 TB system, polymerase chain reaction (PCR) and *FASTPlaque*TB test for detection of *M. tuberculosis* in processed clinical specimens. 40 clinical specimens which were known to be smear positive for AFB and 48 clinical specimens which were known to be smear negative were included in this study. All of the specimens were processed using the N-acetyl-L-cysteine and NaOH digestion-decontamination procedure. In detecting *M. tuberculosis* in smear positive samples, data for BACTEC460 system and *FASTPlaque*TB test gave 87.5% agreement while the agreement between PCR and *FASTPlaque*TB test was found to be 92.1%. In case of smear negative samples, the agreement between BACTEC460 system and *FASTPlaque*TB test was found to be 91.6% while the agreement between PCR and *FASTPlaque*TB test was 95.6%. *FASTPlaque*TB test enables rapid detection of *M. tuberculosis* and has the advantage of being a non-radiometric method. It is a safe and simple system and does not require exogenous gas or radioactive elements. Thus, it may be used for detection of mycobacteria in laboratories desiring a non-radioactive method.

1-06

RAPID DIAGNOSIS OF TUBERCULOSIS IN VARIOUS CLINICAL SPECIMENS BY THE AUTOMATED ROCHE COBAS AMPLICOR *MYCOBACTERIUM TUBERCULOSIS* TEST

Öngüt G[1], Öğünç D[1], Çolak D[1], Ünlü F[1], Öğüş C[2], Gültekin M[1], Vural T[1]
Akdeniz University, School of Medicine,
Departments of [1]Microbiology and Clinical Microbiology,
[2]Pulmonary Diseases, Antalya, TURKEY

The aim of this study was to determine the usefulness of the COBAS AMPLICOR *Mycobacterium tuberculosis* (MTB) PCR assay in diagnosing tuberculosis in various clinical specimens. A total of 46 respiratory specimens, 168 nonrespiratory specimens, and four specimens of unknown origin collected from 200 patients with suspected tuberculosis were subjected to acid-fast bacilli smear, the COBAS AMPLICOR MTB test and culture which was considered as the reference method. Of 218 specimens tested, 202 were negative and two were positive by all three tests. Of the additional four culture-positive specimens for mycobacteria, two were smear-negative but COBAS AMPLICOR MTB positive, two were smear and COBAS AMPLICOR MTB negative. There was one specimen that was negative by smear and culture, but positive by COBAS AMPLICOR MTB test and this specimen was collected from a patient with confirmed clinical diagnosis of pulmonary tuberculosis. Nine smear-positive specimens were found to be negative by both culture and COBAS AMPLICOR MTB test. When COBAS AMPLICOR MTB test results were compared with culture results and with confirmed clinical diagnosis of tuberculosis, the sensitivities, specificities, positive and negative predictive values for the COBAS AMPLICOR MTB test were 66.7%, 99.5%, 80% and 99.0%; 71.4%, 100%,100%, and 99.0% respectively. The results of this study establish the utility of the COBAS AMPLICOR MTB test for the diagnosis of tuberculosis in respiratory and nonrespiratory specimens and this test has the advantage of providing a rapid and specific diagnosis of tuberculosis within hours, in contrast to the the weeks required for culture. However COBAS AMPLICOR MTB test should be used in conjunction with the culture.

1-07

RAPID DETECTION OF STREPTOMYCIN RESISTANCE ASSOCIATED WITH *RPSL* CODON 43 MUTATION IN *MYCOBACTERIUM TUBERCULOSIS* STRAINS BY USING REAL-TIME POLYMERASE CHAIN REACTION AND FLUORESCENCE RESONANCE ENERGY TRANSFER

Erdem N[1], Rota S[2], Kocagöz T[3]
[1]Numune Hospital, Emergency Service,
[2]Gazi University, School of Medicine, Department of Microbiology and Clinical Microbiology,
[3]Hacettepe University, School of Medicine, Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

Resistance to streptomycin (STR) is due to point mutations in either the ribosomal S12 protein gene (*rpsL*) or the 16S rRNA gene (*rrs*) in *Mycobacterium tuberculosis* (*M. tuberculosis*). The majority of point mutations is associated with STR resistance occur at codon 43 of the *rpsL* gene. The most common mutation is an AAG→AGG change in codon 43 resulting in a Lys→Arg substitution. In this study, mutations in *rpsL* gene codon 43 in *M.tuberculosis* strains were investigated by real-time PCR and Fluorescence Resonance Energy Transfer (FRET) methods. Twenty five *M.tuberculosis* strains which were already determined to be STR-resistant by resistance-ratio method using Middlebrook 7H10 medium, showed two different melting curve patterns by FRET probes. While 16 of 25 STR-resistant strains and 47 STR-sensitive strains had similar melting curve patterns when compared to control strain *M.tuberculosis* H37Ra, nine of them had quite different melting curve patterns. Thus it was possible to determine STR resistance in these nine strains by PCR-FRET method. DNA sequencing of the *rpsL* genes of randomly selected six STR-resistant strains and the control strain were performed to confirm the results of PCR-FRET method. Concordant results were obtained in all of those seven strains. Mutation was observed at codon 43 in all of the strains which were determined to be STR-resistant *M.tuberculosis* strains by PCR-FRET method. All of the mutations at amino acid 43 were due to a transition changing of the Lys codon to an Arg codon (AAG→AGG). Strains which had similar melting peaks like to *M.tuberculosis* H37Ra had no mutation at codon 43. This pilot study shows that PCR-FRET method which is very easy to perform, doesn't require any post-PCR sample manipulation and STR resistance associated with codon 43 mutation can be determined within about 40 minutes.

1-08

EVALUATİON OF GEN-PROBE *MYCOBACTERİUM TUBERCULOSİS* DİRECT TEST FOR PULMONARY AND EXTRAPULMONARY SPECİMENS

Esen N[1], Sevinç C[2], Kırdar S[1], Gündüz A T[1]
Dokuz Eylül University, School of Medicine,
[1]Department of Microbiology and Clinical Microbiology
[2]Department of Respiratory Medicine
İzmir, TURKEY

The performance of the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test (MTD; Gen probe, San Diego.Calif) for rapid diagnosis of pulmonary and extrapulmonary tuberculosis was evaluated by testing 106 clinical specimens from patients suspected of having mycobacterial infections. From 106 clinical specimens 65 were pulmonary and 41 were extrapulmonary samples. The golden standart for the diagnosis of tuberculosis was considered to be the combination of culture results with clinical and/or radiological findings. Gen-Probe MTD was interpreted as positive or negative on the basis of recommended cut-off values. The sensitivities, specificities, and positive and negative predictive values of Gen-Probe MTD were 100%, 92.1%, 86.7%, and 100% for respiratory; 90.9%, 90.0%, 76.9%, and 96.4% for nonrespiratory specimens respectively. It is concluded that Gen-Probe MTD is a rapid, sensitive and specific method for the detection of *M.tuberculosis* in both respiratory and nonrespiratory specimens.

1-09

SDS-PAGE INVESTIGATION OF SENSITIVE AND RESISTANT *MYCOBACTERIUM* SPECIES TO ANTI-MYCOBACTERIAL AGENTS ISOLATED IN DIFFERENT GEOGRAPHIC AREAS

Rota S[1], Akyar I[1], Bozdayı G[1], Gümüşlü F[2], Ceyhan İ[2]
[1]Gazi University, School of Medicine, Department of Microbiology and Clinical Microbiology,
[2] Institute of Refik Saydam Hıfzısıhha, Reference Laboratory for Tuberculosis, Ankara, TURKEY

The aim of this study was to separate the whole cell proteins of 99 *M.tuberculosis* strains by SDS-PAGE and investigate the correlation between the protein band patterns both with antibiotic susceptibility patterns and geographic distributions of the strains. Samples were run by SDS-PAGE and protein bands were analysed by densitometer following silver staining. Mod, median, range values were used for statistics.
In conclusion, no correlation was found between protein band patterns and antibiotic susceptibility of the strains.

1-10

PATIENT WITH TUBERCULOSIS ARTHRITIS, PREVIOUSLY DIAGNOSED AS "SYNOVIAL SARCOMA"

Özyurt M[1], Kuşkucu M[2], Ardıç N[1], Kurukuyu T[1], Çiftçi F[3], Kocagöz T[4]
[1]GATA Haydarpaşa Training Hospital, Department of Microbiology and Clinical Microbiology
[2]GATA Haydarpaşa Training Hospital, Department of Ortopedics
[3]GATA Haydarpaşa Çamlıca Chest Diseases Hospital
[4]Diomed A.Ş. Department of R&D,
İstanbul, TURKEY

Tuberculosis can cause infection in all types of tissues and organs in the body. According to the data published by World Health Organization (WHO), tuberculosis arthritis is a rare clinical form of the disease and constitutes 6.6% of extrapulmonary tuberculosis cases. After the onset of symptoms, the establishment of diagnosis, may take from 3 months up to 4 years. *M. tuberculosis* is the most common etiological agent in tuberculosis arthritis. In this reported case who is a 24 years old male patient, the isolated etiological agent was also *M. tuberculosis*. The diagnosis of "synovial sarcoma" was first made after radiological examination of the joint and the chest, the latter of which was normal. Erythrocyte sedimentation rate was 98mm/hour, PPD was negative and biopsy specimen examination was reported as "granulomatous caseification necrosis". Acid fast bacilli was negative by microscobic examination, *M. tuberculosis* was grown from the affected right knee joint, in both radiometric culture system (BACTEC TB 460, Beckton Dickinson Instrument System, Spark, MD, USA) and on Löwenstein Jensen Medium. The isolate was identified to be belonging to *M. tuberculosis* complex group by Polymerase Chain Reaction and Restriction Enzyme Analysis (Dio-Mycotype All, Diomed A.Ş., İstanbul, Turkey) and by Transcription Mediated Amplification (Mycobacterium Direct Test, Gen-Probe, San Diego, CA, USA). Although the isolate was tested to be susceptible to classical antimycobacterial drugs, it was not possible to obtain a clinical improvement by standard tuberculosis treatment regimen. Addition of ofloxacin (400mg b.i.d) to therapy greatly improved the healing. This case shows the importance of microbiological examination in the management of tuberculosis arthritis and also indicate that fluoroquinolones may be important in the treatment, especially when a response to standard regimen is not obtained.

1-11

RAPID DIFFERENTIATION OF MYCOBACTERIA BELONGING TO *M. TUBERCULOSIS* COMPLEX AND OTHER MYCOBACTERIAL SPECIES USING DIO-TK MEDIUM CONTAINING PARA-NITRO-BENZOIC ACID (PNB)

Güney C[1], Adalı G[2], Kocagöz T[2]
[1]Social Insurance Institute, Süreyyapaşa Chest Diseases Hospital, Microbiology Laboratory
[2]Diomed A.Ş. Department of R&D, İstanbul, TURKEY

The major pathogen causing tuberculosis is *M. tuberculosis*. However, infections due to atypical mycobacteria are becoming more common parallel to the increase in the number of immunosuppressed patients and classical antituberculosis drugs are in many case not effective against atypical mycobacteria. Atypical mycobacterial infections are also frequently observed secondary to chronic pulmonary diseases due to other etiology. It is also important to identify the non-pathogenic mycobacteria grown from patient samples in order not to give unnecessary treatment. Two chemicals, para-nitro-benzoic acid (PNB) and p-nitro-alpha-acetylamino-beta-hydroxypropiophenone (NAP) are shown to inhibit the growth of mycobacteria belonging to *M. tuberculosis* complex but not other species of mycobacteria. In this study we have evaluated the efficiency of Dio-TK Medium in rapid differentiation of mycobacteria by adding PNB. The most suitable concentration for PNB was determined to be 750µg/ml which allowed the growth of *M. tuberculosis* H37Ra but inhibited 24 different mycobacterial species assayed so far, which do not belong to *M. tuberculosis* complex group. Dio-TK Medium containing PNB is a practical tool to differentiate mycobacterial species in a short period of time, which is essential for choosing the appropriate treatment regimen.

1-12

MOLECULAR CHARACTERIZATION OF AN ADHESIN OF A RESPIRATORY PATHOGEN, *MORAXELLA CATARRHALIS*

Turan T[1], Ahmed K[1], Tazebay UH[1], Wada A[2], Hatakeyama T[4], Hirayama T[2], Nagatake T[3]

[1]Bilkent University, Department of Molecular Biology and Genetics, Ankara, TURKEY

Nagasaki University, Institute of Tropical Medicine [2]Dept. of Bacteriology, [3]Dept. of Internal Medicine, Faculty of Engineering [4]Dept. of Applied Chemistry, JAPAN

Moraxella catarrhalis is one of the most important causes of respiratory tract infection, conjunctivitis and otitis media. Attachment of this bacterium to human pharyngeal epithelial cells has been shown to be the initial step of pathogenesis. In the attachment mechanism, bacterium expresses adhesin molecules on its surface, which binds with the complementary receptor present on the host cell. Therefore studies on attachment mechanism are important to understand the pathogenesis of infection as well as to design preventive strategies for this infection.
Recently, we isolated a 55 kDa protein from the surface of *M. catarrhalis* and determined the *N*-terminal amino acid sequence. The search of current databases showed that there is no homologue of this protein. We determined that this protein is not glycosylated. In addition, heat and mercaptoethanol treatment did not change the molecular weight of the protein. By Western blotting we found that patients with *M. catarrhalis* infection produce antibody against the 55 kDa protein in their sputum. In this study, we have shown that this protein, when given exogenously, inhibits the attachment of *M. catarrhalis* to host cells and the monoclonal antibody against this protein also inhibits the attachment. In order to clone the gene encoding the 55 kDa protein, we prepared a genomic DNA library and screened the library with the same monoclonal antibody. We identified several clones expressing this protein on *E. coli*. Now we are progressing satisfactorily to sequence this gene. In future, the gene can be mutated to better understand the role of the protein during colonization and infection. Since this surface protein is immunogenic in humans, it might be a potential vaccine candidate for *M. catarrhalis*. Moreover since this protein has no homology with other proteins, it can be used as a marker of *M. catarrhalis*.

1-13

PSEUDO-OUTBREAK OF *BURKHOLDERIA CEPACIA* IN PATIENTS UNDERGOING BRONCHOSCOPY

Gülay Z, Biçmen M, Mutlu D
· Dokuz Eylül University, School of Medicine,
Department of Microbiology and Clinical Microbiology, İzmir, TURKEY

Objective: To investigate the clonal relationships and the source of *Burkholderia cepacia* isolates which were obtained from endotracheal aspirate and bronchoalveolar lavage specimens.
Methods: During 2 weeks period, in 12 bronchoscopic specimens obtained from 11 patients, *B. cepacia* growth was detected. The isolates were identified by API 20NE (bioMerieux, France). The colony counts were below the infection threshold (max. 10^4 cfu/ml). As *B. cepacia* is a rare isolate in our hospital and the patients were mostly outpatients, this was thought to be a pseudooutbreak. In order to find the source, enviromental cultures were taken from the bronchoscopy equipment and other materials used during bronchoscopy. The clonal relationship between the clinical and environmental isolates were investigated by ERIC-PCR.
Results and conclusion: *B. cepacia* was isolated from 0.9 % saline and the aritmal solution prepared with the contaminated saline. All of the clinical and enviromental isolates shared identical ERIC-PCR patterns. *B. cepacia* growth was ceased when the solutions were changed and prepared according to proper infection control practices.

1-14

GENOTYPİNG OF NOSOCOMİAL İSOLATES OF METHİCİLLİN-RESİSTANT *STAPHYLOCOCCUS AUREUS* (MRSA) İN CUMHURİYET UNİVERSİTY HOSPİTAL BY PULSED-FİELD GEL ELECTROPHORESİS (PFGE) AND REPETİTİVE SEQUENCE-BASED (REP)-PCR

Öztop AY[1], Pınarbaşı H[2], Kocagöz S[3], Bakıcı MZ[1], Bakır M[4]
Cumhuriyet University Faculty of Medicine
Departments of [1]Microbiology and Clinical Microbiology, [2] Biochemistry,
[4] Infectious Diseases and Clinical Bacteriology, Sivas, TURKEY
[3] Hacettepe University, Faculty of Medicine, Department of Infectious Diseases,
Ankara, TURKEY

Nosocomial infections caused by MRSA strains have become a major problem in most hospitals. Typing of MRSA strains is necessary for proper investigation of sources and spread of these strains. Several DNA-based methods can be used for genotyping of MRSA. PFGE is accurate, reliable, and has high differentiative ability for typing MRSA, but it requires a specific technique and is time-consuming. Rep-PCR has been suggested as an easy and fast method for monitoring the epidemiology of nosocomial MRSA infections. In this study, we present the results of genotyping of the isolates of MRSA which were isolated from hospitalized patients in Cumhuriyet University Hospital in Sivas by using both PFGE and Rep-PCR. The 43 nosocomial isolates and five control strains (unrelated strains) were tested in this study. PFGE was able to classify the genotypes of clinical isolates into one type and eight subtypes. Rep-PCR banding patterns of these strains were not significantly different from each other. However, control strains could be differentiated by Rep-PCR. The comparison of differentiative ability of these two methods revealed that Rep-PCR can be used to distinguish unrelated epidemic strains of MRSA isolated from nosocomial infections.

1-15

EPIDEMIOLOGICAL ANALYSIS OF *STAPHYLOCOCCUS AUREUS* NASAL COLONIZATION IN HOSPITAL STAFF

Güdücüoğlu H[1], Ayan M[2], Berktaş M[1], Durmaz R[2], Bozkurt H[1], Bayram Y[1]
[1]100. Yıl University,Faculty of Medicine,
Department of Microbiology and Clinical Microbiology, Van, TURKEY
[2] İnönü University, Faculty of Medicine,
Department of Microbiology and Clinical Microbiology, Malatya, TURKEY

The present study was conducted to asses the epidemiological relation of *Staphylococcus aureus* isolates that colonise the nostrils of hospital staffs. Two nasal swabs were taken from each of 327 personnel with a three-weeks interval. After culturing on blood agar for overnight, probable staphylococcal isolates were identified and subjected to tube coagulase test. *S. aureus* nasal carriage was defined in 56 (17.1%) subjects with a positive culture result for both sampling time. Two isolates were methicillin-resistant confirmed by *mec-A* gene. Antibiotyping and arbitrary-primed polymerase chain reaction (AP-PCR) with M13 primer were used for typing of the strains. Antibiotyping distinguished seven types and three subtypes, and 75% of the isolates were clustered in one group. AP-PCR, in contrast, identified 12 distinct patterns with 12 variants. A specific profile was not found among the isolates obtained from the personnel in a particular clinic. These results indicate that antibiotyping has poor discrimination power and the heterogeneity among the nasal *S aureus* strains in the hospital personnel is high.

1-16

ANALYSIS OF THREE OUTBREAKS DUE TO *KLEBSIELLA* SPECIES IN A NEONATAL INTENSIVE CARE UNIT

Ayan M, Kuzucu Ç, Durmaz R, Aktaş E, Çizmeci Z
İnönü University, Faculty of Medicine,
Department of Microbiology and Clinical Microbiology , Malatya, TURKEY

This study was aimed to investigate the epidemiology of 43 Klebsiella isolates obtained from three outbreaks in a neonatal intensive care unit. The first outbreak was observed in January 1999 and 13 *K. pneumoniae* strains were isolated from blood cultures (7), tracheal aspirates (4), urine (1), and incubator (1). The second outbreak in June 2000 included 10 *K. oxytoca* strains, 9 from blood cultures, and 1 from a tracheal aspirate. The third outbreak in October 2001 included 20 *K. pneumoniae* isolates from blood cultures (5), tracheal aspirates (6), urine (2), throat cultures (6), and incubator (1). All isolates were identified by using API 20E. Antibiotic susceptibility patterns and arbitrary-primed polymerase chain reaction (AP-PCR) based fingerprinting performed with M13 primer were used to type the strains. Typing procedures yielded three antibiotypes and three genotypes among the isolates of the first outbreak, three antibiotypes with one subtype and three genotypes in the second outbreak, and two antibiotypes and two genotypes in the third outbreak. The rates of epidemiological relationship confirmed with both methods were 53.7%, 50%, and 85% of the strains of the first, second and third outbreaks, respectively. The concordance between the results of two typing procedures was 74.4 %. Our results indicate that three outbreaks due to Klebsiella sp. do not relate to each other.

1-17

ANALYSIS OF AN OUTBREAK DUE TO *CHRYSEOBACTERIUM MENINGOSEPTICUM* IN A NEONATAL INTENSIVE CARE UNIT

Tekerekoğlu MS[1], Durmaz R[1], Ayan M[1], Çizmeci Z[1], Akıncı A[2]
İnönü University, Faculty of Medicine,
[1]Department of Microbiology and Clinical Microbiology,
[2]Department of Pediatrics, Malatya, TURKEY

The aim of this study was to describe the epidemiological and clinical features of an outbreak due to *Chryseobacterium meningosepticum.* During a 11-day period, the outbreak was observed among four newborns in a neonatal intensive care unit (NICU) in a teaching hospital. All patients yielded *C. meningosepticum* in their blood cultures, in addition, one was colonized in the throat. Antimicrobial susceptibility assay showed complete resistance to penicillins, cephalosporins, aminoglycosides, imipenem, aztreonam, and tetracycline, and sensitivity to ciprofloxacin and trimethoprim-sulfamethoxazole. All patients were empirically treated with amikacin and meropenem. The neonate who was the first to develop sepsis died before the culture result. When *C. meningosepticum* was identified, antimicrobial therapy was changed to a combination of ciprofloxacin, rifampicin and vancomycin, and three neonates were treated successfully. Environmental screening recovered *C. meningosepticum* from two venous catheter lines and one nutritional solution that was opened by health care staff and used for two neonates. Arbitrary primed polymerase chain reaction and antibiogram typing indicated that all isolates were epidemiologically related. This study demonstrates that rapid selection of appropriate antibiotics is very critical for clinical cure and standard precautions should be reconsidered to limit the spread of this bacterium in the NICU in our hospital.

1-18

IDENTIFICATION OF GROUP A BETA-HEMOLYTIC STREPTOCOCCI WITHIN 24 HOURS, USING A BACITRACIN CONTAINING MEDIA, DIO-BACIT

Altındiş M[1], Aktepe OC[1], Çetinkaya Z[1], Beştepe G[2], Çeri A[3]
Kocatepe University School of Medicine,
[1] Department of Microbiology and Clinical Microbiology,
[2]School of Health Services, Department of Puplic Health,
[3]Emirdağ Goverment Hospital, Division of Pediatrics,
Afyon, TURKEY

Bacitracin susceptibility is the most widely used test in the diagnosis of Group A Beta hemolytic Streptococci (GABS), although there are many microbiological and biochemical tests for this purpose. In this study, a new, ready-to-use media, Dio-Bacit, in a two section plate, that contains 5% sheep blood agar on one side and sheep blood agar with bacitracin (2 µg/ml) on the other side, was used for GABS identification in one day.

Throat specimens obtained from 370 children with pharyngitis complaints (between 3-15 years old, 217 boys, 153 girls) between October'2001 and February'2002 were inoculated to Dio-Bacit plates (Diomed Inc.Turkey), first to one side with 5 % sheep blood agar and then the other side with bacitracin. After an overnight incubation at 37°C, colonies with beta hemolysis on 5% sheep blood agar but no growth with bacitracin, were defined to be GABS. Additionally, those colonies are serologically classified by latex-agglutination (Streptococcal Grouping Kit-Oxoid, UK).

Eighty-one of total 370 (21.9 %) inoculations revealed growth of GABS on Dio-Bacit plates. 72 of them were defined as GABS by Latex test. 38 different specimens revealed beta hemolysis and both sections of Dio-Bacit and 6(15.8 %) of them were detected to be GABS by latex. When compared with latex-agglutination test, we found Dio-Bacit method's sensitivity and spesificity to be 93.5 and 96.9 % respectively.

We suggest Dio-Bacit can be used routinely in the diagnosis of GABS since it is ready-to-use, easy for utilizing and gives results within one day.

1-19

PENICILLIN BINDING PROTEIN ANALYSIS OF PENICILLIN RESISTANT *STREPTOCOCCUS PNEUMONIA* ISOLATED IN HACETTEPE UNIVERSITY HOSPITAL

Yenişehirli G, Pınar A, Şener B, Hasçelik G, Günalp A
Hacettepe University, School of Medicine,
Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

The emergence of penicillin and multi-drug resistant pneumococci has become a global concern. The mechanism of penicillin resistance in *S.pneumoniae* involves production of altered forms of penicillin binding proteins (PBPs) that have decreased affinity for penicillin. The purpose of this study was to examine the DNA restriction patterns of the genes pbp 2b and 2x to evaluate the genetic relatedness of 103 penicillin intermediate (n:88) or resistant (n:15) pneumococci isolated in our hospital.
The antimicrobial susceptibilities of the isolates against penicillin, erythromycin, cefaclor, cefotaxime, chloramphenicol, tetracycline, ciprofloxacin, rifampin and vancomycin were determined by the agar dilution methodology according to the NCCLS guidelines.
The tested strains were initially serotyped by the Quellung reaction. The pbp 2b and 2x genes were amplified from chromosomal DNA by PCR. Then the purified pbp DNA was digested with the restriction enzyme *Hinf I* and the fragments were seperated on agarose gel by electrophoresis.
The most common five serotypes were determined to be serotypes 19, 23, 9, 14 and 6 respectively. Pbp 2b gene analysis revealed 5 different profiles while pbp2x gene analysis divided the 103 pneumococci into 12 distinct types. The isolates were further differentiated into 22 groups by a combination of pbp2b and 2x gene analysis, and 44 groups when antimicrobial resistance patterns were combined with the pbp gene analysis results.

It can be concluded that the existence of the common PBP gene fingerprinting and similar antimicrobial resistance patterns suggests that some of the resistant pneumococci exhibit a clonal spread in our country. This possibility of clonal dissemination will further be studied by "Pulsed Field Gel Electrophoresis".

1-20

IRIP (IMMUNORECEPTOR-INTERACTIVE PROTEIN) INDUCES PHOSPHORYLATION OF LYN AFTER IL-5 STIMULATION IN EOSINOPHIL LYSATE

Cen O[1], Stafford SS[2], Alam R[2]
[1]Harran University, School of Science and Literature, ŞanlıUrfa, TURKEY
[2]The University of Texas Medical Branch, Galveston, TX, USA

Eosinophils are important granulocytes in the pathogenesis of asthma, allergic disorders, and parasitic infections. IL-5 is the most important cytokine for differentiation, survival, and activation of eosinophils. IL-5 receptor (IL-5R) is a heterodimer of a unique □ and a common □c subunit. Lyn is a prominent Src family tyrosine kinase in eosinophils and is associated with IL-5R. We have previously reported the cloning of IRIP as an IL-5R□ interacting protein in the yeast two-hybrid system. IRIP is associated with IL-5R□ and Src family tyrosine kinases. We have structurally established that this association is through SH2 and SH3 domains which led to the activation of Lyn kinase. Our objective was to demonstrate the importance of IRIP in the activation of Lyn kinase using co-immunoprecipitation and kinase assay methods. The result we report here is that IRIP immunoprecipitated from IL-5-stimulated, but not that from non-stimulted eosinophil lysate, induced activation of Lyn kinase. In conclusion, these along with our previous data imply that IRIP may switch between 'on' and 'off' states and that IL-5 stimulation may dictate an 'on' state. IRIP may prove to be an important target for therapeutic intervention in asthma, allergic and parasitic disorders.

1-21

A VISUAL BIOINFORMATICS TOOL: VITAL

Engin D
Hacettepe University, School of Medicine,
Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

Visualization and interactive analysis of biological data is critical for rapid and accurate interpretation. Many computer based analysis packages and internet based programs have been introduced to fill the gap between the databases, analysis tools and the human perception. VIsual Tool for ALigned sequences (VITAL) is a Microsoft Windows based software package that was built to arrange, analyse and annotate the output of multiple sequence alignment (MSA) programs like Clustal. The graphical user interface of the program allows instantaneous visualization of the translation of the aligned nucleic acid sequence in 6 frames. This feature is especially useful is assessing the impact of mutations on phenotype. VITAL is also able to search motifs and restriction sites without being affected from the gaps introduced by MSA programs. Standart C++ programming language was used for the core functionality of the program. However, Microsoft Windows platform specific graphical user interface elements were put together using C++Builder 4 to make it possible to port the application to Linux platform in the future.
VITAL is a bioinformatics tool with a graphical user interface. In addition to being a front end for MSA programs, VITAL performs motif searches, translation of aligned nucleic acid sequences and annotations to ease the pre-laboratory work of molecular biologist.

DAY 2

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2-01

SEROLOGY AND POLYMERASE CHAIN REACTION (PCR) RESULTS FOR *HELICOBACTER PYLORI* AND *CHLAMYDIA PNEUMONIAE* IN ATHEROSCLEROTIC PATIENTS

Pınar A[1], Öç M[2], Akyön Y[1], Farsak B[3], Us D[1], Tokgözoğlu L[4], Böke E[2]
Hacettepe University, School of Medicine,
Departments of [1]Microbiology & Clinical Microbiology, [2]Cardiovascular Surgery, [4]Cardiology,
[3]Ankara Numune Hospital Department of Cardiovascular Surgery, Ankara, TURKEY

H. pylori (HP) and *C. pneumoniae* (CP) have been claimed to be associated with the development of atherosclerosis. As the second part of our previous study, we determined the presence of the DNA of these bacteria in atherosclerotic vesels of patients independent from the first study group. The difference between the previous and this study is that we aimed to evaluate the correlation of serology with PCR results for current study.

Materials and Methods: A total of 78 consecutive patients undergoing coroner artery bypass grafting, carotid endarterectomy and surgery of the abdominal aorta for atherosclerotic vascular disease were included in the study. Thirty-three specimens from the atherosclerotic lesions, which were accepted as the patient group and 45 specimens from healthy segments of left internal mammary artery (LIMA) and macroscopically healthy regions of the assending aorta, accepted as the control group, were excised. PCR was used to show the microorganisms DNA in specimens. Serological results were obtained by ELISA kits.

Results & conclusion: The PCR and ELISA results are shown in the table. In this study, there was no significant correlation between PCR and ELISA results ($p>0.05$). In countries like Turkey, HP prevelance is very high, (84 %, at blood donors), therefore Ig G positivity can not be accepted as a marker of atherosclerosis. This study confirms that the presence of HP and CP in vessel wall does not correlate with serology. The presence of HP and CP in nonatherosclerotic segments is a novel ınding. Chronic, persistent infection with these microorganisms may lead to atherosclerosis late on in life.

	HP PCR (+)	CP PCR (+)	HP Serology	CP Serology
Control segment	20.0 %	17.7 %	93.3 %	6.1 %
Atherosclerotic segment	42.4 %*	6.1 %	100 %	3.03 %

* $p< 0.05$

2-02

INVESTIGATION OF *CHLAMYDIA PNEUMONIAE OMPA* GENE DNA IN VASCULAR SEGMENTS OF ATHEROSCLEROTIC AND NON-ATHEROSCLEROTIC PATIENTS, BY POLYMERASE CHAIN REACTION

Koçyıldırım E[1], Açıkgöz ZC[2], Öç M[3], Tuncer S[4] , Karapınar K[1], Sarıgül A[1], Yücel E[1]

[1]Social Insurance Institute, Ankara Training Hospital, Department of Cardiovascular Surgery

[2]Fatih University School of Medicine, Department of Microbiology and Clinical Microbiology

[3]Hacettepe University, School of Medicine, Department of Cardiovascular Surgery,

[4]Metis Biotechnology, Ankara, TURKEY

The possible association between atherosclerosis and some microorganisms has been widely discussed in the recent decade. In this context, the mostly investigated pathogen is *Chlamydia pneumoniae,* due to the presence of high antibody titers against it in atherosclerotic patients, and also due to the presence of *C.pneumoniae* antigens, DNA's or elementary bodies in atherom plaques by electron microscopy, immunocytochemical staining, polymerase chain reaction (PCR), and even by culture. In the present study, by using nested-PCR, we tried to show *C.pneumoniae* DNA in 25 atherom plaques obtained by endarterectomy from atherosclerotic patients, and 25 vascular tissue samples obtained from aortas of non-atherosclerotic patients undergone cardiac surgery for aortic valve replacement. A 207 base pair sequence in *ompA* gene of *C.pneumoniae* was selected as the amplification target. In 14 of the atherom plaques (56%) and in 2 of the nonatheromatouse tissue samples (8%), amplifications with expected DNA size, were observed. There was a statistically significant difference between the two groups ($p<0.05$). Our results are similar to most of the previous reports, however to our knowledge, this is the first study in which the control samples (nonatheromatous vascular tissues) were obtained from different, non-atherosclerotic patients; not from healthy vascular segments of the same atherosclerotic patients, as it was in the previous studies.

2-03

THE RAPID DIAGNOSIS OF BRUCELLOSIS WITH POLYMERASE CHAIN REACTION

Yalınay Çırak M[1], Hızel K[2], Külah C[1], Karakuş R[2], Türet S[1]
Gazi University Faculty of Medicine,
[1]Department of Microbiology and Clinical Microbiology,
[2]Department of Clinical Microbiology and Infectious Disease, Ankara, TURKEY

Brucellosis is a worldwide zoonosis and an important morbidity cause in humans and animals. The laboratory diagnosis of brucellosis is based on the isolation of organism or serological methods which have the disadvantages of prolonged duration of incubation and specificity of tests respectively. The sensitivities of the serological tests range from 65 to 95%, but their specifities in areas where brucellosis is endemic are low because of the high prevalence of antibodies in the healthy population. Moreover most serological tests can produce cross-reactions with other bacteria.
Amplification of DNA by polymerase chain reaction (PCR) is currently used to diagnose several infectious diseases caused by fastidious or slowly growing microorganisms. In this study clinical diagnostic value of PCR was evaluated in brucellosis. 71 active brucellosis patients were included to the study. 38 out of 71 (53.5%) patients were found to be positive by semi-nested PCR.
In conclusion, diagnostic application by PCR in brucellosis seems to be a good alternative compared to conventional methods by means of its rapidity. However low detection limit in blood samples and increased risk of carry-over contamination must be evaluated further.

2-04

A CASE OF BRUCELLA ARTHRITIS DIAGNOSED BY POLYMERASE CHAIN REACTION

Hızel K[1], Yalınay Çırak M[2], Altunçekiç A[1], Külah C[2]
Gazi University Faculty of Medicine,
[1]Department of Clinical Microbiology and Infectious Disease,
[2]Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

Brucellosis is a systemic infection and osteoarticular manifestations of this disease are reported in many patients. Most cases of brucella arthritis are monoarticular and rarely seen in elderly patients. We report a case of brucellosis diagnosed by polymerase chain reaction (PCR), in a 55 years old woman with bilateral knee arthritis. She was admitted to the hospital with fever and pain on both of her knees on March 2001. She had a history of consumption of unpasteurised dairy products and had been treated for acute brucellosis two years ago. Fifteen days ago she had complaints of gastroenteritis. On the day of admission her knees were painful, red and swollen. Examination of the synovial fluid revieled white blood cell count of 12 000/mm^3 and a protein content of 4.2 g/dl. Microscopic analysis revealed predominance of polymorphonuclear leucocytes and no microorganisms. The culture of this specimen did not yield any bacteria. Serum brucella agglutination test was positive at 1/40 titer. She was diagnosed to have reactive arthritis and ofloxacine therapy was initiated, but no clinical improvement was observed. The synovial fluid sample was analysed by PCR for the presence of brucella, and found to be positive. Upon the confirmation of diagnosis of brucellosis, rifampisin and doxycylcine were started and the patient's symptoms resolved progressively. It can be concluded that PCR can be used in the diagnosis of brucella infections with high reliability.

2-05

CTX-M TYPE EXTENDED SPECTRUM BETA LACTAMASE IN A CLINICAL *SHIGELLA SONNEI* ISOLATE

Açıkgöz ZC[1], Gülay Z[2], Göçer S[1], Biçmen M[2], Gamberzade Ş[1]
[1]Fatih University School of Medicine,
Department of Microbiology and Clinical Microbiology, Ankara, TURKEY
[2]Dokuz Eylül University School of Medicine,
Department of Microbiology and Clinical Microbiology, İzmir, TURKEY

Antimicrobial resistance due to the extended spectrum beta lactamases (ESBL) has become a worldwide problem. Until now, various types of ESBL were isolated from several genera of Gram negative bacilli, however there are only a few reports about *Shigella species* producing ESBL, and this is the first report from Turkey. A *S.sonnei* isolate, resistant to cefotaxime and aztreonam, susceptible to ceftazidime, was recovered from a stool sample of a 7 year-old child. Identification of the isolate was confirmed via the three different automatic identification systems in addition to two different antiserum sets. In the double disk synergy test, cefotaxime and aztreonam disks exhibited plus inhibition zones with synergy of amoxicillin-clavulanate disk, but ceftazidime did not. It was shown by an E-test that there was no difference between ceftazidime and ceftazidime-clavulanate MICs. ESBL production of the isolate was confirmed by isoelectric focusing (pI: 7, 7.6, 8.5), and by polymerase chain reaction. SHV, TEM and CTX-M type primers were used to amplify the ESBL-responsible DNA sequence, and only by the CTX-M primers (P1: 5'CGC TTT CGC ATG TGC AG 3'; P2: 5' ACC GCG ATA TCG TTG GT 3'), an amplification occured. Sequencing analysis of the enzyme still continues. Emergence of ESBL-producing *Shigella* strains should be considered as a warning about erroneous and widespread use of antibiotics, especially third-generation cephalosporines.

2-06

MOLECULAR EPIDEMIOLOGY OF MULTIDRUG-RESISTANT *ACINETOBACTER BAUMANNII* ISOLATES FROM HOSPITALIZED PATIENTS IN A MEDICAL CENTER

Ayan M, Durmaz R, Aktas E
İnönü University, Faculty of Medicine,
Department of Microbiology and Clinical Microbiology, Malatya, TURKEY

The aims of this study were to investigate clonal identity among the *Acinetobacter baumannii* isolates in a teaching hospital during a 18-month period and to compare the differentiation ability of antibiotyping, arbitrary-primed polymerase chain reaction (AP-PCR) based fingerprinting performed with M13 and DAF4 primers, and pulsed-field gel electrophoresis (PFGE) of genomic DNA after digestion with Apa I. During this period, a total of 52 *A. baumannii* was isolated. Typing studies were performed on the 38 strains obtained from 36 patients with available epidemiological, clinical and demographic data. Of these 38 strains, 22 were obtained in three outbreaks. The first outbreak affected 9 patients in the pediatric ward, and the latter two affected 6 and 7 patients in orthopedic surgery ward with a six-months interval. All strains were susceptible only to meropenem and imipenem. Antibiotyping, AP-PCR and PFGE confirmed that 68%, 77% and 91% of the outbreak isolates were epidemiologically related, respectively. Based on the PFGE results, discrimination power of the antibiotyping and AP-PCR were 58% and 76.3%, respectively. Our results indicate that a good standardized AP-PCR protocol can be used for the initial screening of clonal identity among the outbreak strains.

2-07

REGULATION OF CARBAPENEM SYNTHESIS GENES IN *ERWINIA CAROTOVORA* SUBSPECIES *CAROTOVORA*.

Tınaz G, Arıdoğan B
Süleyman Demirel Üniversity, Cunur Campus, Department of Biology, Isparta, TURKEY

Carbapenems are a group of β-lactam ring-containing antibiotics which tend to be resistant to the clinically encountered β-lactamase enzymes and are effective against both Gram-positive and Gram-negative bacteria. So far more than 40 naturally occurring carbapenems have been identified, many of which are produced by actinomycetes and particularly *Streptomyces* species. Strain GS101 of the bacterial phytopathogen, *Erwinia carotovora* subspecies *carotovora* (*Ecc*), produces the simple β-lactam antibiotic, (5R)-carbapen-2-em-3-carboxylic acid (carbapenem). In *Ecc*, carbapenem production is subject to control by a regulatory mechanism called "quorum sensing". This system relies on two major components a small diffusible signalling molecule *N*-(-3-oxohexanoyl)-L-homoserine lactone (OHHL) which accumulates in a population density-dependent manner and a transcriptional activator protein. Because there is little known about the control of carbapenem antibiotic production, the major aim of this study was to use gene fusion techniques to analyse any/all environmental and physiological factors that effect antibiotic synthesis in *Erwinia*.A novel carbapenem regulatory gene (*carJ*) was identified via Tn*phoA*'-2 transposon mutagenesis. In addition to the analysis of genetic factors affecting carbapenem production, physiological studies demonstrated that carbapenem synthesis is affected by various environmental factors, such as temperature and different carbon sources. In this study it was shown for the first time that carbapenem production is under control of multiple physiological, environmental and genetic factors.

2-08

MODIFIED MEDIA FOR ISOLATION OF *H. PYLORI* FROM MOUTH SAMPLES

Akyön Y[1], Young KA[2], Rampton DS[3], Hardie JM[2], Feldman RA[4]
[1]Hacettepe University, School of Medicine,
Microbiology & Clinical Microbiology Department, Ankara, TURKEY
St. Bartholomew's & London Hospital School of Medicine & Dentistry,
Departments of [2]Oral Microbiology, [3]Gastrointestinal Science Research Unit,
[4]Epidemiology & Medical Statistics , London, UK.

It is difficult to detect *H.pylori* (HP) in mouth specimens because of flora normally present in the mouth. Aim: This study was designed to improve detection of HP by inhibiting growth of oral flora, for this purpose, we studied dyspeptic patients using Brain Heart Infusion (BHI) agar with 7% horse blood containing HP selective supplement (HPSS), nalidixic acid (NA) and colistin (C). Materials and Methods: Dental plaque samples, were collected in BHI broth. To the broth, 10^8 cfu/ml of HP NCTC 11637 suspension was added. From this spiked sample 10 fold dilutions were prepared to 10^4 cfu/ml. These were inoculated onto a HPSS medium and five HPSS+NA+C media. The susceptibility of the media was tested by the inoculation of 35 gastric biopsy HP strains. 31 oral swab and dental plaque samples were also inoculated on this media before and after endoscopy. Results: HP was not cultured from the spiked samples on HPSS agar, but it was detected on the HPSS+NA+C agar. This agar inhibited most of the oral flora except *Capnocytophaga spp.* HP(11637) was detectable in all of the spiked dilutions. All of the 35 gastric biopsy strains could be detected in 10^8 cfu/ml dilution. For the dilutions 10^7,10^6,10^5,10^4cfu/ml the growth percentage of HP was 94.3, 91.4, 82.9, 80 % respectively. In the studied 31 patient 12 of them had positive cultures for their gastric samples. HP could not be isolated from mouth samples, before and after endoscopy, in the media. Conclusion: The failure to identify viable HP in oral samples if they were present could be because there were less than 10^4 cfu/ml of HP present, or because excretion is intermittent.

2-09

HYDROGEN GAS IS AN ESSENTIAL GROWTH FACTOR FOR *HELICOBACTER PYLORI*

Doruk Engin
Hacettepe University, School of Medicine,
Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

It is generally accepted that *Helicobacter pylori* (HP) is microaerophilic, although the optimal atmospheric conditions for growth of this organism has not been clearly defined. Commercial hydrogen producing – oxygen reducing kit systems are superior than ascorbic acid based systems in supporting growth of HP.
In an effort to assemble a reliable and cheap system to grow HP, an airtight chamber was equipped with CO_2 – input and gas output valves to displace the air inside. An analytical O_2 sensor was used to monitor the O_2 concentrations. An electrolysis tank was installed into the chamber system (HCS) to generate H_2.
In order to assess the role of H_2 in growth, HP strains were cultured and incubated simultaneously in an airtight plastic jar (PJ) and in the HCS. CampyGen CN35 (Oxoid, UK) kit was used in both HCS and PJ to constitute a microaerophilic atmosphere. H_2 was produced in the HCS by electrolysis of Na_2SO_4. Following 72 hours of incubation, the growth characteristics of the cultures and microscopic appearance of the cells were compared.
Afterward, the assembly of the HCS was completed upon connection to CO_2 tube to displace air until the desired level of O_2 (4-5%) is reached. H_2 was produced by electrolysis.
Either CampyGen or air displacement sufficiently reduced the O_2 tension in the HCS which produced confluent growth and predominantly spiral cells. In contrast, PJ yielded poorly grown cultures composed largely of coccoidal cells.
As a conclusion, H_2 appears to be an essential growth factor for HP along with CO_2. HCS is a novel system that provides appropriate O_2 concentration and H_2 to support excellent growth of HP.

2-10

SEROLOGICAL AND MOLECULAR TYPING OF *HELICOBACTER PYLORI* FOLLOWING ERADICATION

Salih BA[1], Abasıyanık MF[1,4], Sarıbaşak H[1], Hüten O[2], Sander E[2]
[1]Fatih University, Faculty of Science, Department of Biology, Microbiology Unit, İstanbul, TURKEY
SSK Samatya Hospital, [2]Department of Pathology and
[3]Department of Gastroenterology, İstanbul, TURKEY
Gebze High Technology Institute [4]Department of Chemistry, Biochemistry Unit, Gebze, TURKEY

Helicobacter pylori eradication heals gastritis and prevents the recurrence of peptic ulcers. The aim was to determine IgG antibody level and genotypes of *H. pylori* after therapy. Materials and Methods: 11 patients (age: 29-66) 9 with duodenal ulcer and 2 with gastric ulcer were given 1g amoxycillin, 500 mg clarithromycin and 30 mg lansoprazole b.i.d. for two weeks. Four biopsies from the antrum were obtained for *Campylobacter*-like-organism (CLO), PCR, culture and histopathology before and one-year after therapy. Also a serum sample for ELISA and immunoblotting was collected from each patient before and on the 1st, 2nd and 12th months after therapy. Results: All patients were *H. pylori* positive by CLO, serology, histopathology and PCR before treatment. The ELISA results were as follows: there was an average 4%, 11% and 40% decrease in the IgG titer at first month, second months and twelfth months after therapy. PCR results showed cagA gene in 10 (91%) and vacA-s1 gene in 8 (73%) of 11 patients. All patients were negative at one month by CLO and PCR but became positive in one patient after twelve months. Histopathology results showed a decrease in the prevalence of *H. pylori* of 55% and 75% after one month and twelve months following therapy. Conclusion: A 40% decline in antibody titer indicates eradication of *H. pylori*. The majority of patients possessed cagA gene together with vacA-s1 gene.

2-11

SEROLOGICAL DETECTION OF *HELICOBACTER HEILMANNII* IN CHILDREN

Akyön Y[1], Bennedsen M[2], Yüce A[3], Gürakan F[3], Özen H[3], Saltık İN[3], Koçak N[3], Wadström T[4], Andersen LP[2].
Hacettepe University, School of Medicine,
Departments of [1]Microbiology & Clinical Microbiology, [3]Pediatric Gastroenterology, Ankara, TURKEY,
[2]Rigshospitalet, Department of Clinical Microbiology, Copenhagen, DENMARK,
[4]Lund University, Department of Medical Microbiology, Lund, SWEDEN

Helicobacter heilmanni has been shown in histological sections of the gastric tissue and it has recently been cultured from human gastric mucosa. The aim of this study was to detect *H. heilmannii* specific antibodies by ELISA in children with various gastrointestinal complaints.
Materials & Methods: Sixty-four serum samples were collected from children, (age range: 1-16, 41 female, 23 male) who admitted to Hacettepe University, İhsan Doğramacı Children's Hospital. *H. heilmannii* antigen for ELISA: Whole sonicate of *H. heilmannii* (8.5µg/well). Absorption antigen: Sonicate plus whole cell suspension of *H. pylori* strain CH-20249. Control sera were obtained from seven known *H. heilmannii* positive donors, 11 donors seropositive for *H. pylori* 11 donors known to be *Helicobacter* negative. The ELISA was specific for *H. heilmannii,* as there was no cross-reaction with the following bacteria: *H. pylori, E.coli, P. aeruginosa, C. jejuni* or *S. aureus* as defined by western blotting.
Results: Cut off level was defined as, ELISA unit (Eu)<0.7 are negative, Eu>1.4 are positive and 0.7<Eu>1.4 are in the grey zone. According to the cut off levels three (4.7 %) of the children were seropositive for *H. heilmannii*, two of these patients were also positive for *H. pylori*. Three of the patients were borderline; again two of them positive for *H. pylori* and the rest were accepted negative. *H. pylori* was found positive in 46 (71.9 %) children.
Conclusion: The 4.7 % seropositivty for *H. heilmannii* is at least 10 times higher than normally reported from histological investigations. The explanation for this could be problems of finding the scarce bacteria and non-persistent infections.

2-12

DETECTION OF *HELICOBACTER PYLORI* IN DENTAL PLAQUE AND SALIVA SAMPLES WITH NESTED POLYMERASE CHAIN REACTION

Yalınay Çırak M[1], Külah C[1], Çırak E[2], Türet S[1]
[1] Gazi University, Faculty of Medicine,
Department of Microbiology and Clinical Microbiology,
[2] Self employed dentist, Ankara, TURKEY

Helicobacter pylori is a gram-negative, micro-aerophilic bacterium which is recognised as being an aetiological agent of chronic active gastritis and peptic ulcer disease, and also has been associated with gastric cancer. The precise mode of transmission and the natural reservoir for *H. pylori* are unknown. Dental plaque and saliva have been implicated as possible sources of *H. pylori* infection. The aim of this study was to investigate the presence of *H. pylori* in the dental plaque and saliva by polymerase chain reaction (PCR). 23 dyspeptic patients and 10 randomly selected patients with no dyspeptic complaints as control group were included to the study for detecting the presence of *H. pylori* in dental plaque and saliva. Totally 33 dental plaque and şaliva samples were amplified with 16S rRNA gene primers by nested PCR. *H. pylori* DNA was detected in 9 (39%) out of the 23 dental samples and 10 (43%) out of the 23 saliva samples. In the control group only 2 (20%) of the dental plaque samples showed to have *H. pylori* and there was no *H. pylori* in the saliva samples. As a result *H. pylori* was present in the oral cavity and dental plaque may be a reservoir for *H. pylori* infection.

2-13

THE *CagA* STATUS OF *HELICOBACTER PYLORI* ISOLATES FROM DISPEPTIC CHILDREN IN TURKEY

Engin D[1], Darka Ertunç Ö[1], Özçay F[2], Demir H[2], Saltık İ[2], Akyön Y[1], Koçak N[2]
Hacettepe University, School of Medicine,
[1]Department of Microbiology and Clinical Microbiology,
[2]Pediatric Gastroenterology Unit, Ankara, TURKEY

Inconsistent reports exist regarding *cagA* status of *H. pylori* isolates and the severity of the mucosal lesions.
We examined 45 *H. pylori* strains isolated from 22 male and 23 female pediatric patients with ages ranging from 6 to 16. *H pylori* strains were grown on brain heart infusion agar supplemented with 7% horse blood. Following 72 hours of incubation, colonies were harvested and bacterial DNA was extracted. Polymerase chain reaction (PCR) primers F1 (5'-GAT AAC AGG CAA GCT TTT GAG G-3') and B1 (5'-CTG CAA AAG ATT GTT TGG CAG A-3') were used to amplify a 348bp internal fragment of *cagA*. The gastroduodenal lesions were assessed visually by endoscopic examination. Table shows the *cagA* status of the isolates and the endoscopic evaluation of the 45 patients.

Endoscopic examination	*cagA* PCR Negative (%)	Positive (%)	Total (%)
Normal	5 (11.11)	5 (11.11)	10 (22.22)
Minimal nodularity, hyperemia	4 (8.89)	6 (13.33)	10 (22.22)
Prominent nodularity, ulcer	11 (24.44)	14 (31.11)	25 (55.56)
Total	20 (44.44)	25 (55.56)	45 (100)

The prevalence of *cagA* in Turkish pediatric patients was %55.56. Although statistically insignificant, patients with endoscopically detectable lesions tend to cluster in the *cagA* positive group. Thus, we were unable to confirm an association between *cagA* status and the severity of gastric and duodenal lesions.

2-14

HELICOBACTER PYLORI CagA STATUS IN ASYMPTOMATIC SUBJECTS IN TURKEY

Abasıyanık MF[1,2], Tunç M[1], Salih BA[1]
[1]Fatih University, Faculty of Science,
Department of Biology, Microbiology Unit, İstanbul, TURKEY
[2]Gebze High Technology Institute, Department of Chemistry, Biochemistry Unit, Gebze, TURKEY

Approximately half of the world population is infected with *Helicobacter pylori* particularly in the developing countries. The aims of the study were to detect *H. pylori* prevalence in asymptomatic Turkish subjects, to correlate the prevalence of *H. pylori* with age, sex, education, socioeconomic status, smoking, non-steroidal anti-inflammatory drugs intake, and marital status and to detect antibodies to CagA and other *H. pylori* antigens by immunobloting. Methods. Three hundred and nine asymptomatic subjects 1-82 years of age (average 31) with no previous history of epigastric pain were selected randomly from the Turkish population. Serum samples were obtained and stored at -20°C. Results. The ELISA test detected IgG anti-*H. pylori* antibodies in 216 (70%) of 309 asymptomatic subjects (185 (60%) males, 124 (40%) females). Infection rates of 42% in subjects <10 years of age, 55% in 10-19 years, 66% in 20-29 years, 78% in 30-39 years, 79% in 40-49 years, 91% in 50-59 years, 100% in 60-69 years and 80% in those >70 years of age. Subjects >45 years of age had significantly higher antibody responses than those <45 years. *H. pylori* infection was significantly higher in subjects with low socioeconomic status than those with middle or high class. No difference in sex, smoking, NSAIDs intake and education was seen among subjects. Immunoblots revealed immunoreactive bands to CagA (116kDa) in 33 (84%) of 40 samples examined. Conclusion. *H. pylori* is highly prevalent in the asymptomatic Turkish subjects. Forty two percent of subjects under 10 years were found infected and increased to >66% in older age. Antibodies to CagA antigen were highly prevailing in these subjects.

2-15

HELICOBACTER PYLORI CagA AND VacA GENE VARIABILITY IN DYSPEPTIC PATIENTS

Sarıbaşak H[1], Sander E[2], Salih BA[1]
[1]Fatih University, Faculty of Science, Department of Biology, Microbiology Unit
[2]SSK Samatya Hospital, Department of Gastroenterology, İstanbul, TURKEY

The *H. pylori* cagA gene was considered as a marker for increased virulence and the vacA-s1, but not s2 was found to be associated with in vitro cytotoxin activity, peptic ulcer disease and the presence of cagA gene. The aim of the study was to detect cagA and vacA genes in gastric biopsies from patients with peptic ulcer diseases (PUD) and gastric cancer using PCR and to determine its correlation with such diseases.

Materials and Methods: Sixty-seven patients attending the endoscopy unit complaining of dyspepsia and abdominal pain were selected randomly. Two biopsies were obtained from the antral part of the stomach (one for *Campylobacter*-like organism (CLO) test, one for direct PCR).

Results: CagA gene was detected by PCR in 9 (42.8%) of 21 patients with gastritis, 30 (88.2%) of 34 with PUD and 11 (91.6%) of 12 with cancer patients. vacA signal sequence genotype s1a was detected in 19 (90%), 24 (86%) and 11 (92%) of gastritis, PUD and gastric cancer patients respectively. On the other hand, all cancer (100%) and PUD (100%) patients that had s1a genotype, were cagA positive whereas out of 19 vacA-s1a gastritis patients only nine (47.3) had the cagA gene.

Conclusions: These results indicate that cagA and vacA-s1a are strongly correlated with PUD and gastric cancer, however vacA-s1a is also strongly correlated with gastritis. The presence of cagA in all vacA type s1a PUD and gastric cancer patients suggests that cagA is strongly associated with vacA-s1a.

2-16

RFLP-PCR PATTERN OF *HELICOBACTER PYLORI* CagA GENE: ASSOCIATION WITH PEPTIC ULCER AND GASTRIC CANCER

Sarıbaşak H[1], Sander E[2], Salih BA[1]
[1]Fatih University, Faculty of Science, Department of Biology, Microbiology Unit
[2]SSK Samatya Hospital, Department of Gastroenterology, İstanbul, TURKEY

A method utilizing RFLP-PCR in the *H. pylori* genes is widely used to differentiate strains. The aim of the study was to evaluate the digestion pattern of *H. pylori* cagA by two different restriction endonucleases.
Materials and Methods: 21 cagA+ patients (age: 30-54) 8 with gastritis, 9 with peptic ulcers and 4 with gastric cancer. Two biopsies from the antrum were obtained for *Campylobacter*-like organism (CLO) and PCR. RFLP was done using restriction endonucleases *EcoRI* and *HaeIII*.
Results: *EcoRI* digestion revealed the same pattern among twenty patients. For *HaeIII*, there were two different patterns of digestion fragments. 4 out of 8 gastritis, 4 out of 9 peptic ulcer and 1 out of 4 gastric cancer (GC) patients showed the same pattern. A second pattern was observed in 1 out of 9 PUD and 1 out of 4 GC patients.
Conclusion: *EcoRI* digestion revealed the same pattern among the *H. pylori* strains examined, which indicates similarity between the strains however *HaeIII* digestion revealed variability among these strains.

2-17

DETECTION OF *HELICOBACTER PYLORI* FROM GASTRIC BIOPSY SAMPLES FROM PATIENTS WİTH UPPER GASTROİNTESTİNAL SYSTEM COMPLAINTS AND COMPARISON OF FOUR DIAGNOSTIC METHODS

Uraz G[1], Aşkın S[1], Tuncer C[2]

Gazi University, [1]Faculty of Science and Arts, Department of Biology,
[2]Faculty of Medicine, Department of Gastroenterology, Ankara, TURKEY

H. pylori infection is a worldwide gastrointestinal system disease. For the diagnosis of this disease microbiological, histopathological, molecular methods are used. The aim of our study was to compare, culture, microscobic examination, rapid urease test and ELISA tests, in the diagnosis of *H. pylori*.

Materials and Methods: A total of 70 patients were included in the study. From each patient biopsy material and blood were obtained. Culture was performed in microaerophilic conditions, for microscobic examination Gram stain was performed, rapid urease test was performed for each biopsy material, IgG was detected by ELISA test.

Results: *H. pylori* was positive in 34 (48 %), 37 (53 %), 40 (57 %), 56 (80 %) of the patients by culture, Gram stain, rapid urease test and ELISA respectively. In 27 (38 %) patients all four tests were found positive. According to endoscopical examination 45 of the patients were diagnosed as gastritis, 11 of them as duodenitis, three of them as oesephagitis, one as gastric ulcer, four as duodenal ulcer and six patients had normal findings. Although these six patients had normal findings, two of these patients had all four tests positive for *H. pylori*. In our study group *H. pylori* was mostly detected in patients whom were diagnosed as gastritis.

2-18

USING PCR AND REAL-TIME PCR (LIGHT CYCLER) FOR DIAGNOSIS AND FOLLOW UP OF INVASIVE ASPERGILLOSIS IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES AND TRANSPLANTATION

Işık N[1], Badur S[1], Poynton CJ[2]
[1]University of İstanbul, İstanbul Medical Faculty,
Department of Microbiology and Clinical Microbiology, İstanbul, TURKEY
[2]University of Wales, College of Medicine, Department of Haematology, Wales, UK.

Purpose: In modern medicine, for early diagnosis of infections, tests that have high specificity and sensitivity should be preferred. For this reason, especially for patients with hematological malignancies and transplantation, that have high mortality and morbidity rates, some molecular biological techniques are coming in to use today for differential diagnosis and follow-up of invasive fungal infections. In the coming years it will become easier to early diagnose and also to plan optimal treatments, by using these techniques for diagnosis of fungal infections.
Materials and methods: In this study, we made fungal DNA isolation from blood samples, which were taken from patients, in immunodeficiency state, with hematological malignancies and transplantation. For PCR, we used primers specific to *Aspergillus* species. By using Real-time PCR samples from 20 patients, which were found positive by PCR, the amount of DNA was measured. In Real-time PCR, we used *Aspergillus* colonies as our standard, and for this purpose, SDA petri dishes were incubated for 72 hours at 30°C and; 10^1-10^6 CFU/ml serial dilutions were made by using hemocytometry. We performed DNA isolation and PCR from these dilutions. *Aspergillus* species-specific products giving one band of 500 bp, were fixed in 2% agarose gel. We measured the DNA amounts by Real-time PCR by using Sybr Green I. Standard dilution series and extraction samples were studied at the same time by Real-time PCR. Measurement of the DNA quantities in the separate samples, one at the time of diagnosis and the other after 15 days were interpreted by the Light Cycler system.
Results: According to the first extraction results, the results for five patients showed 10^5-10^6 CFU/ml; for 10 patients, 10^4-10^5 CFU/ml; for four patients, 10^3-10^4 CFU/ml; and for one patient, 10^1-10^2 CFU/ml. The results for the samples taken after 15 days for two patients were $>10^6$ CFU/ml; for two patients, 10^5-10^6 CFU/ml; for 11 patients, 10^4-10^5 CFU/ml and for five patients, 10^3-10^4 CFU/ml.
Conclusion: These findings showed that Real-time PCR is a new, specific and a sensitive method among the other quantitative PCR systems. This method was also quicker than the other quantitative PCR systems. Currently, the investigators prefer this method because, it is important for early diagnosis of infections, for early treatment of patients who are under risk of developing these infections, and for follow-up of treatment results.

2-19

DIFFERENTIATION OF *ASPERGILLUS* AND *CANDIDA* SPECIES BY USING RFLP METHOD

Işık N[1], Mills KI[2]
[1]University of İstanbul, İstanbul Medical Faculty,
Department of Microbiology and Clinical Microbiology, İstanbul, TURKEY
[2]University of Wales, College of Medicine,
Department of Haematology, Wales, UK.

Purpose: Today, it is known that *Aspergillus fumigatus* and *Candida albicans* species are most pathogenic species for immunocompromised patients. Fungal infections, especially for immunocompromised patients, have high morbidity and mortality rates. Because of this, for early diagnosis of infectious agents and for planning of species-specific treatment protocols; using quick, specific and sensitive methods are very important.
Materials and methods: In this study, we managed rapid interpretation of different fungal sequencing analysis by using the RFLP method with species-specific enzymes for four different agents isolated from fungal infections in the Department of Haematology, University of Wales, College of Medicine. In the RFLP method, DNA was fragmented by using one or more restriction enzymes and these fragments were separated by agarose gel electrophoresis and made visible by ethidium bromide. According to these visible bands and their comparison, the results were interpreted. The RFLP results were dependent on good selection of species-specific restriction enzymes. These enzymes generally cut the amplified DNA into 4-6 nucleotide fragments. Density of bands in gel were proportional with the DNA fragment length. In this study, SDA petri dishes were incubated for 72 hours at 30°C and then colonies from the culture were taken and DNA extraction was done. After this procedure, PCR was performed. The products were purified by Qiagen kits; these products were incubated for 2 hours at 37°C with Bcr-I and Hpy CH4 IV restriction enzymes.
Results: After the RFLP interpretation of bands at 3% agarose gel, *A.niger*, *A.fumigatus*, *C.albicans* and mixed species (*A.niger* and *A.fumigatus*) were detected.
Conclusions: Although the RFLP method is easily proceded, it is difficult to interpret the results due to the complex band patterns. So, only Candida species are studied in general with this method till today. In this study, using only one restriction enzyme differentiated *C.albicans*, *A.niger*, *A.fumigatus* and mixed species rapidly and reliably.

2-20

SENSITIVITY OF PHENOTYPIC METHODS IN DIFFERENTIATION OF *CANDIDA DUBLINIENSIS* FROM *CANDIDA ALBICANS*

Ertunç Darka Ö, Sancak B, Arıkan S, Hasçelik G.
Hacettepe University Medical School, Department of Microbiology and Clinical Microbiology, Ankara, Turkey

Background: *Candida dubliniensis* is a recently described *Candida* species closely related to *Candida albicans* due to its phenotypic features of ability to produce germ tubes and chlamydospores. To have a better understanding of the role of *C. dubliniensis* in clinical infections, it is essential to identify this species rapidly and accurately. We had previously identified *C. dubliniensis* isolates by phenotypic characteristics of inability to grow at 45°C and formation of abundant chlamydospores on cornmeal agar and we confirmed the identification of these isolates by using polymerase chain reaction (PCR), suggesting that phenotypic tests were very specific in identification of *C. dubliniensis*. In the present study, we investigated the sensitivity of these phenotypic tests in definitive differentiation of *C.albicans* from *C.dubliniensis*.
Methods: 120 clinical isolates that were presumptively identified as *C.albicans* by their ability to produce germ tubes, grow at 45°C and to produce few and singly arranged chlamydospores on cornmeal agar, were screened by PCR for verification of the identification. *C. albicans* ATCC 64548 and *C. dubliniensis* CD36 type strain were included as control strains. PCR was performed by using the previously described *C.dubliniensis*-specific (DUBF and DUBR) and *C. albicans*-specific (PSpA1 and PSpA2) primer pairs. DUBF and DUBR are complementary to sequences within the ACT1-associated intron sequence of *C.dubliniensis* and yield an amplimer of 288 bp whereas PSpA1 and PSpA2 code for *C.albicans* NTS region and yield an amplimer of 684 bp.
Results: All 120 yeast isolates that were phenotypically identified as *C.albicans* yielded amplimers specific for *C. albicans*.
Conclusions: Phenotypic features of ability to grow at 45°C and to produce few, singly arranged chlamydospores are very sensitive in exclusion of *C. dubliniensis* and identification of *C. albicans*. These tests offer a rapid and accurate way of differentiation of these two *Candida* species.

2-21

IMPORTANCE OF POLYMERASE CHAIN REACTION FOR DETECTION OF CONGENITAL *TOXOPLASMA GONDII*

Bozdayı G[1], Kuştimur S[1], Biri A[2], Özensoy Töz S[3], Dayangaç N[3], Gürüz Y[3]
Gazi University, School of medicine,
[1]Department of Microbiology and Clinic Microbiology,
[2]Department of Gynecology and Obstetrics, Ankara, TURKEY
[3]Ege University, School of Medicine, Department of Parasitology,
İzmir,TURKEY

Toxoplasma gondii infection acquired during pregnancy can lead to infection of the fetus, which may cause fetal loss or lesions that usually involve the brain and the eyes. The aim of this study was to detect the DNA of *Toxoplasma gondii* in the amniotic fluid of pregnant women by using a two step PCR method to investigate the intrauterine infection. *Toxoplasma* IgM, IgG antibodies, IgG avidity and PCR of amniotic fluid were found to be positive at the first trimester of the pregnancy. At the 16th week of pregnancy, Toxoplasma IgM became negative (ELISA Immuncapture IgM negative),while IgG antibodies were positive (IFAT IgG: 1/128; ELISA IgG 1/1024). Two different primers sets designed for *T.gondii* B1 gene were used for PCR analysis. In addition to direct PCR analysis of the specimen, the amniotic fluid sample obtained at 16th week of pregnancy was also injected into a mouse peritoneum and the peritoneal fluid obtained from the mouse was also tested by PCR. The PCR results of the samples were positive.
In conclusion, PCR detection of *T.gondii* may generate usefull early clinic information in addition to serologic markers.

2-22

CLONING AND SEQUENCE ANALYSIS OF CLINICAL ISOLATES WITH *ENTAMOEBA HISTOLYTICA* AS REFLECTED IN THE Gal/GalNAc LECTIN AND THE SERINE-RICH GENES

Tanyüksel M[1], Beck D[2], Haque R[3], Petri Jr WA [2]
[1]Gülhane Military Medical Academy,
Department of Microbiology and Clinical Microbiology, Division of Parasitology, Ankara, TURKEY
[2]University of Virginia Health System,
Departments of Medicine, Pathology, Microbiology, Charlottesville, Virginia, U.S.A.
[3]International Centre for Diarrhoeal Disease Research, Dhaka, BANGLADESH

The protozoan parasite *Entamoeba histolytica* is a major cause of colitis and liver abscess in humans. The Gal/GalNAc lectin gene has been described as one of the major amebic protein responsible for adherence and virulence to human colonic glycoproteins. The Serine-Rich *E. histolytica* Protein (SREHP), a cell surface protein, is a valuable marker in evaluation of host-parasite interactions. With this study, we investigated genetic differences in the clinical presentations of amebic colitis from Mirpur Camp, Dhaka, Bangladesh by analyzing polymorphism in the Gal/GalNAc lectin and the SREHP genes by nested PCR, cloning, and sequencing and *Alu*I digestion. These lectin gene sequences are compared with the lectin gene from the HM1: IMSS laboratory strain of *E. histolytica*. Interestingly, we observed the distinct DNA banding pattern among intestinal isolates and genetic diversity in the SREHP gene. But, we found slightly genetic diversity in some locuses of the lectin gene. This remarkable degree of genetic conservation of the Gal/GalNAc lectin between isolates concluded that important role in mediating amebic adherence to the host made it a potential target for strain cross-protective and colonization-blocking antibody responses.

2-23

DIFFERENTIATION OF *ENTAMOEBA HISTOLYTICA* AND *ENTAMOEBA DISPAR* SPECIES BY POLYMERASE CHAIN REACTION

Dağcı H[1], Töz Özensoy S[1], Üstün Ş[2], Akarca US[2],Mete H [2], Dinçer Ç[2]
Ege University School of Medicine,
[1]Department of Parasitology, [2]Gastroenterology Department, İzmir, TURKEY

Amoebiasis is an important disease threatening human health and it causes gastrointestinal system complaints, amoebic dysentery and abscesses in different organs. Classically, the agent of amoebiasis is *Entamoeba histolytica*, but currently, it has been proven that the disease can be caused by two different species (*E.histolytica* and *E. dispar*). *E. histolytica* has invasive characteristics and produces serious complaints. However, *E.dispar* leads to asymtomatic infection or mild symptoms. Differentiation of these species is not possible by stool staining technics and can only be achieved by isoenzyme analysis and moleculer methods such as Polymerase Chain Reaction (PCR). In our study, we aimed to differentiate *E.histolytica* and *E.dispar* by PCR in stools from patients which have been diagnosed as *E.histolytica*. Two stool samples which have been shown *Blastocystis hominis* + *Entamoeba coli* + *Iodomoeba butschilii* + *E.histolytica/E.dispar* and *B.hominis* + *E.coli* + E.histolytica/E.dispar by wet-mount examination and Trichrome stain were inoculated into Robinson medium and amoeba were cultured. Culture supernatants were collected and DNA were extracted. Amplification of both the control DNA (*E.histolytica* and *E.dispar*) and the isolated genomic DNA (gene coding 30 000 MR antigen) was performed by PCR and band products of 101 bp which was compatible with *E.dispar* were obtained. In this study, *E.histolytica* and *E.dispar* could definitely be differentiated. These are the preliminary results of the current study entitled "Diagnosis and differentiation of *E.histolytica* and *E.dispar* in stools clinically suspected as ameobiasis".

2-24

DIAGNOSIS OF *CRYPTOSPORIDIUM PARVUM* AND *GIARDIA LAMBLIA* AND DIFFERENTIATION OF GENOTYPES OF *CRYPTOSPORIDIUM PARVUM* WITH REAL-TIME PCR ASSAY

Tanrıverdi S[1], Tanyeli A[1], Köksal F[2], Feng X[3], Widmer G[3]

Çukurova University, School of Medicine,
[1]Department of Pediatric Hematology-Oncology,
[2]Department of Microbiology and Clinical Microbiology, Adana, TURKEY
Tufts University, School of Veterinary Medicine,
[3]Department of Infectious Diseases, MA, USA

Objectives: Cryptosporidial infection is one of the most common non-viral causes of human diarrhea. Waterborne outbreaks of *Cryptosporidium parvum* and *Giardia lamblia* have been documented. The objective of this study is the application of multiplex real-time PCR for *C.parvum* and *G.lamblia* detection and for differentiating among type 1 (anthroponotic) and type 2 (zoonotic) *C.parvum*. Identifying the types of *C.parvum* is relevant to tracking sources of oocysts as the occurence of type 1 oocyst is indicative of contamination by human waste. Traditionally, genotyping of *C.parvum* has relied on PCR-RFLP or sequencing, which are both time-consuming techniques.

Methods: For diagnosis and genotyping of *C.parvum*, the β-tubulin gene was chosen as PCR target; for *G.lamblia* a gene encoding a cyst wall protein. For *C.parvum* genotyping, two fluorescent probes complementary to the type 2 sequence within a polymorphic region of the β-tubulin gene were used. During the annealing phase of each PCR cyclethe probes hybridized to an internal sequence of the amplified fragment. After completion of the PCR procedure, melting curve analysis was performed to determine the temperature at which the hybridization probes were released. The procedure was completed in 35 min.

Results: In experiments using SYBR Green I, a dye which fluoresces when bound to double-stranded DNA, specific PCR amplifications of the *C. parvum* β-tubulin and *G. lamblia* cyst wall protein gene were observed in individual or multiplex amplifications. When probes were used to genotype *C. parvum* oocysts, melting curve analysis was capable of differentiating between *C. parvum* types on the basis of only 2 single-nucleotide polymorphisms between type 1 and type 2 *C. parvum*. The difference in melting temperature between *C. parvum* types was statistically highly significant.

Conclusions: To our knowledge, this is the first application of the real-time PCR for the simultaneous diagnosis of *C.parvum* and *G.lamblia* and for genotyping *C.parvum* isolates. The high specificity and sensitivity of the real time PCR approach suggest that these methods are powerfull tools for environmental monitoring, clinical diagnosis and studies of parasite population dynamics in infected individuals and animals.

DAY 3

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3-01

CLONING AND EXPRESSION OF HBcAg GENE OF HEPATITIS B VIRUS (HBV) IN EUKARYOTIC CELLS

Bulut Y[1], Doymaz MZ[1], Bulut H[2], Özdarendeli A[1]
University of Fırat, Faculty of Medicine,
[1]Department of Microbiology and Clinical Microbiology,
Faculty of Veterinary,[2]Department of Virology, Elazığ, TURKEY

In the present study, cloning and expression of the HbcAg gene in eukaryotic cells are reported. For this aim, initially, Hepatitis B virus (HBV) core antigen gene was placed into pUC19 plasmid. The resulting recombinant plasmid, termed pHBc-1, was used in transformation of *E. coli* cells. Later, HBcAg gene was excised from pHBc-1 and inserted into an eukaryotic expression vector pcDNA3. Afterwards, pcDNA-HBc was transfected into Vero cells. Cells stably transfected with pcDNA-HBc plasmid (Vero-HBc) were selected in geneticin containing culture medium. The presence of HBcAg gene in Vero-HBc cells were again confirmed with PCR assay. Additionally, the expression of HBcAg gene by Vero-HBc cells were tested by Western Immunoblotting assay. The result of immunoblotting assay demonstrated that Vero-HBc cells indeed express a 21 kDa protein reacting with anti-HBcAg antibody.

3-02

GENOTYPING OF TURKISH HEPATITIS B VIRUS ISOLATES BASED ON THE SEQUENCING OF S REGIONS

Külah C, Yalınay Çırak M

Gazi University, Faculty of Medicine,

Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

Variability in hepatitis B virus DNA allowed its classification into seven main genotypes (A-G). The pattern of HBV genotypes varies depending on the geographical region and it has been speculated that viral genotype can influence the clinical course of the disease in infected patients.
Objectives: The aim of this study was to determine HBV genotypes in a group of patients with hepatitis B infection in Turkey where this infection is endemic.
Methods: Fourty-two Turkish patients with HBV infection were included in the study. Genotyping was performed by amplifying the S region of the genome and direct sequencing of PCR products by a commercial Cy 5 /Cy 5.5 Dye Primer Cycle Sequencing Kit based on di-deoxy chain termination method. The sequences were compared with the previously reported sequences in the database bank for each genotype.
Results: According to the comparative analysis of S-sequences of all patients with the published sequences of the genotypes in database bank, all of the fourty-two hepatitis B strains were shown to be related to D genotypic group, subtype ayw.
Conclusion: There are only few data about the prevalent genotypes of HBV in Turkey. Here we report that the main genotype and subtype of HBV in Turkish patients are genotype D and ayw, respectively .

3-03

NUCLEOTIDE AND AMINO ACID DIVERGENCES IN THE CORE PROMOTER AND PRECORE REGION OF GENOTYPE D HEPATITIS B VIRUS IN PATIENTS WITH PERSISTENTLY ELEVATED OR NORMAL ALT LEVELS

Bozdayı AM[1,2], Bozkaya H[1,2], Türkyılmaz AR[1], Erkan Ö[1], Sarıoğlu M[2], Çetinkaya H[2], Karayalçın S[1,2], Yurdaydın C[1,2], Uzunalimoğlu Ö[1]

Ankara University,

[1]Institute of Hepatology,

[2]School of Medicine, Department of Gastroenterology, Ankara, TURKEY

Aim:The aim of the study was to investigate the association of nucleotide and amino acid divergences in both core promoter and precore mutations with liver cell injury (reflected by ALT levels), HBeAg and anti-HBe status and viral replication activity in a large group of Turkish patients with chronic HBV genotype D infection. We postulated that high disease activity may be mediated by an immune response and would correlate with a larger heterogeneity in the core promoter and precore region sequences.

Patients and Methods: In this study, the sequences of the core promoter and the precore region of HBV DNA extracted from 67 patients' samples, all having genotype D and subtype *ayw,* were analyzed. The patients were divided into two groups and four subgroups, according to their HBeAg and anti-HBe status, and ALT profile. Persistently elevated ALT or persistently normal ALT definition was used when serum ALT levels were at least two times the upper limit of normal or stayed within normal limits during the nine months observation period, respectively, on at least three determinations done more than one month apart.

Results: It was found that the nucleotide and amino acid divergences in the core promoter but not in the precore region were higher in patients having persistently elevated serum ALT than in serum ALT normal patients in both HBeAg positive and anti-HBe positive groups ($p<0.05$). The number of T/A and A1896 stop codon mutations did not yield a statistically significant difference between ALT normal and elevated groups. It was also found that 1762-1764 T/A and precore A 1896 mutation existed in 15% and 18% of the HBeAg positive patients, respectively. In anti-HBe positive groups, 1762-1764 T/A and precore A1896 mutation were only detected in 8% and 50% of the patients respectively, and coexisted in 21% of the patients.

Conclusion: Precore A 1896 stop codon mutation seems to play an essential role in the loss of HBeAg in Turkish patients. Nucleotide variability in the core promoter region may be one of the factors linked to hepatitis B disease activity.

3-04

COMPARISON OF CLASSICAL METHOD AND HELIOSIS™ FOR THE EXTRACTION OF HEPATITIS B VIRUS DNA

Bozdayı G, Rota S, Dinç B, Doğan B

Gazi University, School of Medicine,

Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

In this study we aimed to compare two different extraction methods of HBV DNA for PCR detection by using conventional proteinase K-phenol-chloroform method and commercially available extraction kit, Heliosis™ (Viral DNA extraction kit; Metis Biotechnology Ltd., Ankara, Turkey) in the sera of 62 patients. Forty-seven HBsAg positive, 10 negative and 5 HBsAg unknown sera were tested in both HBV extraction assays. Twenty two serum samples were found to be positive, while 33 were negative with both of the methods. Three HBsAg positive sera yielded positive results only by classical method, where four HBsAg positive sera were positive only by Heliosis™ viral DNA extraction kit.

In conclusion, we found that Heliosis™ Viral DNA extraction kit is as sensitive as conventional viral DNA extraction method based on proteinase K digestion.

3-05

YSDD: A NOVEL MUTATION IN HBV DNA POLYMERASE CONFERS CLINICAL RESISTANCE TO LAMIVUDINE

Bozdayı AM[1,2], Uzunalimoğlu Ö[1], Türkyılmaz AR[1], Aslan N[1], Sezgin O[3], Şahin T[3], Bozdayı G[4], Çınar K[2], Bozkaya H[1,2], Schinazi RF[5], Karayalçın S[1,2], Yurdaydın C[1,2]

Ankara University,
[1]Institute of Hepatology,
School of Medicine, [2]Department of Gastroenterology,
Yüksek Ihtisas Hospital, [3]Department of Gastroenterology,
Gazi University, School of Medicine,
[4]Department of Microbiology and Clinical Microbiology,
Ankara, TURKEY
Emory University, School of Medicine,
[5]Department of Pediatrics,
Atlanta, Georgia,USA

Background: The emergence of drug-resistant virus in hepatitis B virus (HBV) patients treated with lamivudine is well documented. In this study, we determined the mutations occurring in the tyrosine-methionine-aspartate-aspartate (YMDD) amino acid motif of the HBV DNA polymerase gene, as well as upstream and downstream of this region, in patients with breakthrough virus during lamivudine therapy.

Patients and Methods: Thirty-one Turkish patients with chronic HBV infection who completed at least 104 weeks of lamivudine treatment were investigated. In this group, 20 patients had HBeAg positive chronic hepatitis B and 11 patients were HBeAg negative and anti-HBe positive. All patients received lamivudine, (150 mg/d), for 104 weeks, with or without 4 months of interferon (IFN) combination. HBV specific sequences were amplified by PCR from sera of patients with breakthrough virus, and the PCR products were directly analyzed by sequencing.

Results: Breakthrough virus was detected in 7 of the 31 patients (22.6%) between 9 and 18 months of therapy. Of the seven patients, 6 were HBeAg positive at baseline and 4 had a double mutation consisting of rtM204V and rtL180M, while 2 had an rtM204I change. In one patient, two base substitutions at rt204; (ATG → AGT; T to G and G to T) leading to a methionine to serine change (YMDD→YSDD) were detected as a novel DNA pol mutation in month 18 of lamivudine treatment. This new variant in addition had rtL180M mutation and a 12 base pairs deletion in the pre-S1 region between nucleotides 43-54.

Conclusion: The results indicate that, in addition to Met→Val and Met→ Ile change in YMDD, Met→ Ser change at rt204 (YMDD→YSDD) associated with rtL180M variant can also emerge during lamivudine treatment, which confers lamivudine resistance in vivo leading to virological breakthrough and ALT increases.

3-06

DEVELOPMENT OF A QUANTITATIVE REAL-TIME DETECTION ASSAY FOR HEPATITIS B VIRUS DNA

<u>Erkan Ö[1]</u>, Türkyılmaz AR[1], Bozdayı AM[1,2]
Ankara University,
[1]Institute of Hepatology,
School of Medicine, [2]Department of Gastroenterology, Ankara, TURKEY

Background: The quantitative determination of hepatitis B virus (HBV) DNA is essential to evaluate the replication activity of the virus. A highly reproducible and sensitive real-time detection assay based on TaqMan technology was developed for the detection of HBV DNA and compared wit a commercially available assay.
Method: Following the extraction of the reference plasma by using commercial spin columns based on silica method, a pair of primers and a double flourescein labeled taqman probe (5' FAM labeling as reporter and 3' TAMRA labeling as quencher) derived from DNA polymerase gene of HBV DNA and a taq polymerase having 5' exonuclease activity were used for the real-time PCR detection of HBV DNA in a real-time PCR instrument (ABI Prism 7000 Sequence Detection System, ABI, US). Real-time PCR method was also compared with HBV Hybrid Capture assay (Digene, US).
Results: Analysis of the amplification plots yielded that the system had a dynamic range from 10^2 to 10^{10} genome per mL. The slope of the standart curve was –3.3 and r^2 value was 0.99. Intra-assay and inter-assay variations of the assay were in the range of the values expected for a clinical test. The results of the real-time PCR showed an excellent correlation with the commercial HBV Hybrid Capture assay (r=0.95).
Conclusion: Real-time PCR can be used for the absolute quantification of HBV DNA with a high sensivity and reproducibility. The real-time PCR method provides very useful clinic information for the values less than 5 pg/mL (10^6 genome/mL) and more than 2000 pg/mL (10^9 genome/mL) which are the dynamic ranges of commercial HBV Hybrid Capture assay widely used in the labs. We concluded that this assay is an excellent altenative for monitoring HBV–infected patients in routine diagnostics and clinical practice.

3-07

QUANTITATIVE DETECTION OF HEPATITIS B VIRUS DNA USING TWO DIFFERENT REAL-TIME PCR METHODS

Eroğlu C[1], Günaydın M[2], Atilla A[1], Esen Ş[1], Bayırlı D[1], Sünbül M[1], Leblebicioglu H[1]
Ondokuz Mayıs University, School of Medicine,
[1]Department of Clinical Microbiology and Infectious Diseases,
[2]Department of Microbiology and Clinical Microbiology
Samsun, TURKEY

The measurement of hepatitis B virus (HBV) DNA in serum has become an important tool to identify chronic hepatitis B patients with viral replication, to follow on therapy and to predict whether antiviral therapy will be successful or not. Several real-time PCR methods have been used in the last few years to quantify serum HBV DNA levels more accurately. The aim of this study was to compare two different real-time PCR methods which based on SYBR Green I and TaqMan technology.
Sixty six serum samples positive for hepatitis B surface antigen were tested. SYBR Green I method is based on getting fluorescence from binding of SYBR Green I to double stranded PCR product. TaqMan method is based on break of fluorophor-labeled probe by exonuclease activity of taq polymerase. Wilcoxon and Chi-square tests were used for statistical analysis.
The ratio of HBV DNA positivity (positive level limit $\geq$ 1000 copy/ml) was found to be 51.5% by SYBR Green I method and 45.5% by Taqman method. The quantitative and qualitative results indicated that there was no statistically meaningful difference between these methods by Wilcoxon and Chi-square test respectively.
As a result, it has been concluded that both methods can be considered to be suitable for routine quantification of HBV DNA because they are easier, rapid and lower detection limit than conventional quantification methods.

3-08

HEPATITIS B VIRUS GENOTYPES AND VIRAL LOAD ANALYSIS IN PRE AND POST VACCINATION SERA FROM CARRIER CHILDREN

Mısırlıoğlu M[1], Kayın E[1], Akman E[2], Tuncer S[1]

[1]Metis Biotechnology Ltd., Ankara, TURKEY

[2]SSK Hospital, İskenderun, TURKEY

Hepatitis B virus (HBV) strains isolated worldwide have been classified into six genomic groups A to F, deduced from genome comparisons and have a characteristic geographic distribution. The efficacy of HBV vaccine to provide seroconversion and persistent antibodies against hepatitis B surface antigen (anti-HBs) has been investigated previously. However, data on effect of the vaccine on viral loads in HBs Ag carriers is lacking. In this study, HBV genotypes and the effect of immunization on viral loads were analyzed in serum samples of HBs Ag carriers obtained through a vaccination program in primary school children at Iskenderun city. HBV DNA S-gene amplification product of 57 HBs Ag positive children were sequenced by using OpenGene automated DNA sequencing system. A phylogenetic analysis was carried out by using those sequences. To understand the effect of vaccination on viral loads, 12 HBs Ag positive children were immunized together with Anti-HBs negative children by using the same schedule and recombinant HBV vaccine dose. HBV DNA quantitations of pre and post vaccination sera were done by simultaneous amplification on Light Cycler real-time polymerase chain reaction (PCR) system together with HBV DNA standards. The phylogenetic analysis showed that all 57 sequences were genotype D with an average heterogeneity of 1.2%. A significant change in HBV DNA titer was not detected between pre and post vaccination serum samples. S-gene sequences suggested the existence of a strong founder viral population as in Mediterranean area, where genotype D predominates. In conclusion, the vaccination applied by using the regular scheme did not effect the viral load. Further investigations are required to reveal whether seroconversion may be obtained by the application of an accelerated scheme and increased doses of recombinant hepatitis B virus vaccine.

3-09

HEPATITIS B VIRUS GENOTYPES AND SUBTYPES, HEPATITIS C VIRUS GENOTYPES AND HEPATITIS DELTA VIRUS GENOTYPES IN TURKISH PATIENTS WITH HEPATITIS VIRUS INFECTIONS

Bozdayı AM[1,2], Aslan N[1], Bozdayı G[3], Türkyılmaz AR[1], Şengezer T[1], Wend U[4], Erkan Ö[1], Bozkaya H[1,2], Gerlich W[4], Yurdaydın C[1,2], Uzunalimoğlu Ö[1]
Ankara University,
[1]Institute of Hepatology,
School of Medicine, [2] Department of Gastroenterology,
Gazi University, School of Medicine,
[3]Department of Microbiology and Clinical Microbiology, Ankara, TURKEY
Justus-Liebig University, School of Medicine, [4]Department of Virology, Giessen, GERMANY

Background: Genotypes may influence the clinical outcome of the disease and display geographic dissimilarities. It seems that it is crucial to determine the occurrence of diverse genotypes and subtypes in definite populations, which may lead to contribute to the clinical settings. The aim of this study is to evaluate the HBV genotypes and subtypes, HCV genotypes and HDV genotypes in Turkish patients with chronic hepatitis in a large cohort of patients.
Patients and Methods: A total of 681 patients with hepatitis B, C and D infection were enrolled in the study. A multiplex PCR and subtype specific ELISA method for HBV subtyping and direct sequencing for HBV genotyping were used in 3 different groups consisted of 83, 71 and 67 patients, respectively. HDV and HCV genotypes were determined by PCR-RFLP in 59 patients with chronic D hepatitis, and 365 patients with chronic C hepatitis and 36 hemodialysis patients with anti-HCV or HCV RNA positivity, respectively. All PCR based studies were further confirmed by direct sequencing.
Results: HBV subtyping by two methods and HBV genotyping yielded that almost 99 % of the patients were subtype *ayw* and genotype D. HDV genotype was 100 % genotype 1. Genotype 1 was the most common genotype of HCV RNA (95%).
Conclusion: The subtype *ayw* and the genotype D of HBV DNA, and the genotype 1 of HDV RNA represent almost 100% of related infections. The genotype 1b of HCV RNA was found to be significantly dominant in Turkish patients.

3-10

THE GENOTYPES OF HEPATITIS C VIRUS IN HAEMODIALYSIS PATIENTS FROM MANISA/TURKEY

Şanlıdağ T, Akçalı S, Özbakkaloğlu B
University of Celal Bayar, School of Medicine,
Department of Microbiology and Clinical Microbiology, Manisa, TURKEY

Objectives: To investigate the genotypes distribution of HCV in haemodialysis patients from Manisa/Turkey.
Methods: Forty-three (% 28.4) out of hundred and fifty-one patients with anti-HCV antibodies assigned to two different haemodialysis units in our region were studied. The presence of anti-HCV antibodies was determined by a third generation enzyme immunoassay (EIA), viral RNA in serum was detected by reverse-transcription polymerase chain reaction (RT-PCR) and genotyping was performed by restriction fragment length polymorphism (RFLP).
Results: Of the forty-three haemodialysis patients, thirty-four (79 %) were RNA negative, and nine (21%) were RNA positive. Genotype was determined in nine patients and the most common genotype was 1b (89 %), and followed by 1a (11 %). The other genotypes were not found.
Conclusion: We showed that the distribution of common genotype 1b, in Turkish haemodialysis patients in our region was similar to the previous studies. Interestingly, there was no relationship between transfusion and HCV infection. This observation together with predominance of type 1b suggest that the infection was more likely originated by nosocomial way.

3-11

THE MUTATIONS IN THE ISDR OF NS5A GENE ARE NOT ASSOCIATED WITH RESPONSE TO INTERFERON (IFN) TREATMENT IN TURKISH PATIENTS WITH CHRONIC HEPATITIS C VIRUS 1a AND 1b INFECTION.

Aslan N[1], Bozdayı AM[1,2], Çetinkaya H[2], Erkan Ö[1], Sarıoğlu M[2], Turkay C[2], Bozkaya H[1,2], Karayalçın S[1,2], Yurdaydın C[1,2], Uzunalimoğlu Ö[1]
Ankara University,
[1]Institute of Hepatology, [2]Department of Gastroenterology, Ankara, TURKEY

Background: A significant association between variations in amino acid sequences resides in between 2209-2248 nucleotides of HCV non-structural 5A (NS5A) gene and response to interferon treatment has been proposed. The aim of this study was to determine whether the amino acid sequence changes in the interferon sensitivity-determining region (ISDR) could be correlated to response to alpha IFN treatment in Turkish patients infected with HCV genotypes 1b or 1a.
Patients and Methods: Thirty-nine patients with chronic hepatitis C virus (35 and 4 patients with genotypes 1b and 1a, respectively) infection, receiving 3x3-5 MU of interferon α-2b for six months were included in the study. Following PCR amplification of the region from pre-treatment serum samples, the products were directly sequenced. The amino acid sequence of NS5A was compared with the published sequence for HCV-J (AA 2209-2248). Mutant type was defined as three or more amino acid mutations, and intermediate type as 1-3 amino acid in this region. Otherwise, they were defined as the wild type. HCV RNA viremia levels were analyzed by branched DNA assay.
Results: Eighteen patients were responders (R; 46%), whereas twenty-one patients were non-responders (NR; 54%). Pretreatment serum viremia levels correlated with response to IFN. Amino acid changes in both R and NR groups did not show any significant difference. Intermediate or wild type strains were detected in both groups.
Conclusion: In this study, we couldn't obtain a significant association between number of amino acid changes in NS5A2209-2248 and response to IFN treatment. In the majority of the patients, it seems that amino acid sequences in the region are well conserved.

3-12

EVALUATION OF INDETERMINATE HCV CONFIRMATION ASSAY RESULTS

Sayıner AA, Sayan M, Abacıoğlu YH
Dokuz Eylül University, Faculty of Medicine,
Microbiology and Clinical Microbiology Department, İzmir, TURKEY

Objective:To evaluate the indeterminate results in two commercial HCV confirmation assays with the magnitude of antibody response and the presence of HCV-RNA.
Methods: Twenty one sera that were indeterminate by either one of the two commercial HCV confirmatory assays (HCV Blot 3.0, Genelabs Diagnostics and Inno-Lia HCV Ab III, Innogenetics) were evaluated for their EIA (Axsym HCV 3.0, Abbott) S/CO value and band patterns of the both confirmatory assays and HCV RNA status.
Results:
Group 1 - HCV Blot indeterminate sera (n=13): Nine were positive by Inno-Lia. Six of them had S/CO value over 26 and were RNA positive. Single core band positivity (n=9) was the most common cause of the indeterminate results, followed by NS5 (n=3) reactivity.
Group 2 - Inno-Lia indeterminate sera (n=8) : All of them were negative by HCV blot. Only one sample was RNA positive with a S/CO value of 1,5 . S/CO of the group was between 1.17-4.5. Single NS3 band reactivity (n=6) was the most prominent cause of the indeterminate results.
Conclusion: HCV Blot assay gave indeterminate result in six sera that were Inno-Lia and RNA positive. Five of these could have been accepted as positive by the HCV blot, if the criteria for positivity included single core band reactivity with ≥2 (+) intensity. The most prominent cause of the indeterminate results was single core band reaction for HCV Blot and NS3 reaction for Inno-Lia.

3-13

COMPARISON OF DIFFERENT KITS FOR DETECTION OF HEPATITIS C VIRUS ANTIBODIES

Altındiş M, Çetinkaya Z, Aktepe OC
Kocatepe University, School of Medicine,
Department of Microbiology and Clinical Microbiology, Afyon, TURKEY

Although there are many kinds of currently available commercial ELISA kits for antiHCV investigations, they may sometimes reveal conflicting intra- or inter-laboratory results. We investigated 84 serum specimens revealing suspected results such as conflicting data on repeats, gray-zone or obscure positivity or negativity by 8 different kits; UBI HCV (Organon bv, NL), Abbott HCV (version 3.0, Abbott Park, USA), Murex antiHCV (4th version, Murex Biotech, SA), Innotest HCV ab IV (4th generation, Innogenetics NV, Belgium), Trinity HCV (3rd generation, Trinity Biotech plc. Ireland), Biochem HCV (version 3, Biochem immunoSystems Inc,Canada), Biokit HCV (Biokit, SA, Spain), and Equipar HCV (Equipar srl, Italy). We performed our tests in full accordance with the recommended instructions and suggestions of the manufacturers and confirmation of all tests were made with InnoLIA ab III update (Innogenetics NV, Belgium) Western Blotting. The table displays the obtained results.

Table. Sensitivity, specificity and predictive value(pv) of anti-HCV tests (n: 84)

HCV tests	Sensitivity	Spesificity	Positive pv	Negative pv
Organon	80	99	80	97
Murex	100	100	100	100
Innotest	100	100	100	100
Abbott	80	99	80	97
Biochem	100	100	100	100
Trinity	100	100	100	100
Biokit	80	99	80	97
Equipar	80	99	80	97

Some of these kits appear to be superior in providing accurate anti-HCV results. However, these data must be supported with large-scale studies. In conclusion all conflicting HCV results need to be confirmed.

3-14

DETECTION OF GBV-C/HGV IN SERUM FROM PATIENTS WITH HEPATITIS C (HCV) AND HEPATITIS B (HBV).

Özdarendeli A[1],Bulut Y[1],Ağçı Toroman Z[1], Doymaz MZ[1], Kalkan A[2], Kılıç SS[2]
University of Fırat, School of Medicine,
[1]Department of Microbiology and Clinical Microbiology,
[2]Department of Clinical Microbiology and Infectious Disease, Elazığ, TURKEY

Hepatitis G virus (GBV-C/HGV) is a newly described RNA virus with a parental route of transmission. HGV is phylogenetically most closely related to HCV. Similarities between HGV and HCV is at the nucleotide sequence level. We undertook to study the prevalance of GBV-C/HGV infection in patients with HBV as well as HCV in Elazığ TURKEY. We studied 80 samples,35 with HCV RNA, anti-HCV positive and 45 with HBV DNA and HBsAg positive. GBV/HGV RNA was detected, by using RT-PCR and nested PCR, in 7 (%20) and 4 (8,88%) the samples with HCV and HBV positive, respectively. We found that both the patients with HBV and HCV superinfected with GBV/HGV. However, we still do not know whether the superinfection with GBVHGV has any impact on liver disease caused by either HCV or HBV.

3-15

PREVALANCE OF TT VIRUS (TTV) DNA IN PATIENTS WITH HEPATITIS B (HBV) AND HEPATITIS C (HCV) INFECTION

Bulut Y, Özdarendeli A, Ağçı Toroman Z, Doymaz MZ
University of Fırat, School of Medicine,
Department of Microbiology and Clinical Microbiology, Elazığ, TURKEY

A novel hepatitis virus, transfusion transmitted virus (TTV), has been isolated from patients with non-A-G hepatitis. Previous studies showed that high prevalance of TTV DNA is common. The aim of this study is to evaluate the prevalance of TTV DNA in patients with HBV and HCV infection. We studied 80 samples, 35 with HCV RNA,anti HCV positive and 45 with HBV DNA and HBsAg positive. TTV DNA was detected in 11 (%38,5) and 9 (%20) the samples with HCV and HBV positive, respectively. After performing semi-nested PCR by using Okomato primers as expected, it was detected high prevalance of TTV DNA in 2 different patient groups.

3-16

A FOLLOW UP STUDY OF CYTOMEGALOVIRUS INFECTION IN A GROUP OF TURKISH RENAL TRANSPLANT RECIPIENTS USING MOLECULAR ASSAYS

Yalınay Çırak M[1], Rota S[1], Maral I[2], Türet S[1], Sindel Ş[3]
Gazi University, School of Medicine,
[1]Department of Microbiology and Clinical Microbiology,
[2]Department of Public Health, [3]Department of Nephrology, Ankara, TURKEY

Objective: The aim of this study was to evaluate the clinical value of two molecular assays for monitoring a group of Turkish renal transplant patients in a follow up study.
Methods: An in-house cytomegalovirus (CMV) nested polymerase chain reaction (CMV-PCR) and a commercial molecular assay the Murex Hybrid Capture CMV DNA assay (HCA) were evaluated for monitoring twenty-four renal transplant patients for six months follow up.
Results: The sensitivities, specificities, positive predictive values, and negative predictive values of nested CMV DNA PCR assay and HCA at the beginning of the study were 70%, 42.9%, 46.7%, 66.7% and 60%, 78.6%, 66.7% and 73.3% respectively. After six months, these rates were found as follows: 80%, 66.7%, 80%, 66.7% for *CMV* PCR and 73.3%, 88.9%, 91.7%, 66.7% for HCA respectively.
Conclusions: In monitoring and predicting CMV infections in renal transplant recipients, not only qualitative but also quantitative assays must be used together in order to decide the preemptive strategies.

3-17

COMPARISON OF CYTOMEGALOVIRUS ANTIGENEMIA ASSAY WITH COBAS AMPLICOR CMV MONITOR IN DIFFERENT PATIENT GROUPS

Çolak D[1], Öğünç D[1], Öztürk F[1], Öngüt G[1], Tuncer M[2], Gura A[3], Ugur A[4], Gölbaşı İ[5], Küpesiz A[6], Gültekin M[1]
Akdeniz University, School of Medicine,
Departments of [1]Microbiology and Clinical Microbiology,
[2]Internal Medicine and Nephrology, [3]Pediatry and Newborn,
[4]Internal Medicine and Hematology, [5]Heart and Vasculer Surgery,
[6]Pediatry and Hematology, Antalya, TURKEY

Antigenemia and quantitative polymerase chain reaction (Q-PCR) are widely used for diagnosis of cytomegalovirus (CMV) diseases. The purpose of this study was to compare antigenemia and CMV Q-PCR results in different patient groups. Blood samples, obtained from 39 solid organ transplant recipients, 12 bone marrow transplant recipients and 11 newborns, were submitted for detection of CMV and were processed by two methods: CMV antigenemia assay (CINAkit, Argene-Biosoft, French) and CMV Q-PCR (Cobas Amplicor CMV Monitor, Roche Diagnostics Systems, Inc, Branchburg, NJ, USA). Of 101 blood samples tested, 22 were positive (antigenemia range 1-3717/2x10^5 leukocytes and median 32.5/2x10^5 leukocytes; Q-PCR range 467-1000000 copies/ml and median 9965 copies/ml) and 72 were negative by both methods. Additional three samples negative by Q-CR were positive by antigenemia assay (range 1-6/2x10^5 leukocytes and median 2/2x10^5 leukocytes) and four samples negative by antigenemia assay were positive by Q-PCR (range 612-17200 copies/ml and median 4165 copies/ml). The evaluation of the results showed a good correlation between the number of CMV copies in plasma and the number of pp65 positive cells ($p<0.001$). In conclusion, CMV antigenemia and Cobas Amplicor CMV Monitor tests are both very convenient assays combining rapidity and simplicity and allow quantitative determinations. The choice between these two assays may rely on their costs and on the experience and familiarity with them. However, using these two assays together enhances the efficacy and performance of the laboratory.

3-18

PRENATAL DIAGNOSIS OF CONGENITAL CYTOMEGALOVIRUS (CMV) INFECTION IN PREGNANT WOMEN WHO UNDERWENT AMNIOCENTESIS

Küçükcan A[1], Yarkın F[1], Evrüke C[2], Özgünen TF[2]
Çukurova University, School of Medicine,
[1]Department of Virology, [2]Department of Obstetrics and Gynecology,
Adana, TURKEY

This study was performed to detect the presence of possible congenital CMV infections among pregnant women who underwent amniocentesis because of various obstetric reasons. Anti-CMV IgG and anti-CMV IgM antibodies were measured, by CMV ELISA (CLARK laboratories, Inc.) in maternal sera. Anti-CMV IgG antibodies were detected 91 (96.8%) of a total of 94 pregnant women and three pregnant women were found to be CMV seronegative, Anti-CMV IgM antibody response were positive in 19 (20.2%). CMV DNA was investigated in amniotic fluid samples by polymerase chain reaction (PCR) and CMV DNA was not found in any of samples. In conclusion, the risk of congenital CMV infection risk was not detected in our study group because of high maternal CMV seropositivity (96.8%).

3-19

THE VALUE OF PCR METHOD IN A DIAGNOSTIC CHALLENGE: A CASE OF ATYPICAL HERPES SIMPLEX VIRUS INFECTION IN THE EYE

Gürelik G[1], Yalınay Çırak M[2], Aydın B[1], Hondur A[1], Hasanreisoğlu B[1]

Gazi University School of Medicine,

[1]Department of Ophtalmology,

[2]Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

Objective: To emphasize the value of PCR method for the diagnosis of Herpes simplex virus (HSV) infection in atypical inflammatory conditions in the eye.
Case presentation: A case of atypical posterior segment involvement of HSV with following tuberculosis meningitis is presented here. The patient was a 31 years old female, complaining of visual loss, pain and redness in the left eye at the first presentation. She had a history of abortus due to candidal infection of the genital tract 20 days before the eye complaints. Mild inflammatory reactions were present in the anterior segment of the eye in contrary to severe posterior segment inflammation revealing papillitis, papilledema, focal retinitis and epiretinal membrane formation with normal systemic findings. Cultures and microscopy showed no specific agent in the humor aqueous sample whereas PCR for HSV was surprisingly positive in the vitreous and epiretinal membrane specimens showing HSV infection supported by a positive blood sample for HSV-1 IgM. Vision was improved from counting fingers to 20/40 after vitrectomy and oral acyclovir therapy. After two months, the patient was hospitalized with presumptive diagnosis of menengitis and increased intracranial pressure, and *Mycobacterium tuberculosis* was the agent responsible for the intracranial findings. The patient was successfully treated with antituberculosis medications and a ventriculoperitoneal shunt was performed. An immunosuppresive condition was suspected due to repeated infections. However immunological evaluation showed no immunosuppresive state.
Conclusions: HSV can play a role in nonspecific, atypical ocular inflammations and the existence of HSV DNA can be detected in ocular fluids and membranes by PCR which is an important diagnostic tool for HSV.

3-20

DETECTION OF HERPES SIMPLEX VIRUS DNA IN CORNEA SAMPLES USING POLYMERASE CHAIN REACTION

Yalınay Çırak M[1], Sarı A[2], Külah C[1], Aydın B[2], Hondur A[2], Akata F[2]

Gazi University School of Medicine,

[1]Department of Microbiology and Clinical Microbiology,

[2]Department of Ophtalmology, Ankara, TURKEY

Herpesviruses are involved in the pathogenesis of many ocular diseases, including keratitis. The rapid and accurate diagnosis of herpetic infections has become increasingly important with the rising incidence of immunosuppressed patients.

Objective:To evaluate the value of the polymerase chain reaction (PCR) technique as diagnostic assay for detecting herpes simplex virus (HSV) in the cornea of patients undergoing keratoplasty.
Methods: The corneas of 19 patients, 6 with a history of herpes keratitis and 13 with non-herpetic corneal disease were investigated using PCR.
Results: HSV DNA was detected in 67% of the cornea samples of patients with herpes keratitis and in 23% of corneas of patients with no history of herpes keratitis.
Conclusion: PCR seems to be a sensitive method for detecting HSV in the cornea. The results demonstrate that PCR may be of potential use in obtaining a diagnosis in scarred corneas without a history of herpetic keratitis as well. The detection of HSV infection can also help in planning preemptive therapy.

3-21

DETECTION OF HERPES SIMPLEX VIRUS DNA BY POLYMERASE CHAIN REACTION IN OCULAR FLUIDS AND MEMBRANES

Gürelik G[1], Yalınay Çırak M[2], Aydın B[1], Külah C[2], Hasanreisoğlu B[1]

Gazi University School of Medicine,

[1]Department of Ophtalmology,

[2]Department of Microbiology and Clinical Microbiology, Ankara, TURKEY

Objective: To assess the prevalence of herpes simplex virus (HSV) DNA in ocular fluids and membranes obtained from patients undergoing vitreoretinal surgery.
Methods: Paired serum and ocular fluid (aqueous humor, vitreous) or membrane samples obtained between January 2001 and January 2002 from 41 patients without uveitis or retinitis, were analysed for herpes virus DNA by PCR. Out of 41 patients, 19 were operated for retinal detachment, 14 were diabetic patients with proliferative diabetic retinopathy, and 8 were with miscellaneous pathologies (1 traumatic vitreous hemorrhage, 2 hypertensive vitreous hemorrhage, 2 macular hole, 2 vitreous condensation as a sequel of old posterior uveitis and 1 choroidal neovascular membrane). Serologic testing for immunoglobulin G levels were also tested for herpes virus.
Results: A total number of 58 (11 aqueous humor, 37 vitreous and 10 vitreal-epiretinal membrane) intraocular fluid-membrane specimens were collected from 41 patients with no clinical evidence of active viral chorioretinitis. 12 of 58 specimens (or 11 of 41 patients) gave positive results for herpes simplex DNA (1 of them aqueous, 8 vitreous and 3 membrane specimens). Positive immunuglobulin G levels were also found for herpes virus in 12 patients. 3 of 12 (25 %) serum Ig G positive patients had a positive HSV DNA with PCR for any of their ocular specimens and 3 of 11(27 %) patients who had a positive HSV DNA with PCR for ocular specimens had a positive serum HSV IgG. Only 3 patients have both positive serum IgG and HSV DNA in ocular samples.
Conclusions: The existence of HSV DNA can be detected by PCR in ocular fluids and membranes although there is no evidence of active ocular inflammation in the eyes and no direct concordance with the serum HSV IgG positivity. Molecular methods will be useful and efficient for evaluating the ocular specimens and the ocular HSV infections. These results may show a high rate of latent herpes virus infections that persist in ocular tissues which requires further investigation.

3-22

SCREENING FOR HERPES SIMPLEX VIRUS BY PCR IN AN OBSTETRICS AND GYNECOLOGY OUTPATIENT CLINIC IN TURKEY

Bozdayı G[1], Biri A[2], Rota S[1]
Gazi University, School of Medicine,
[1]Department of Microbiology and Clinical Microbiology,
[2]Department of Gynecology and Obstetrics, Ankara, TURKEY

The aim of this study was to determine the incidence of the Herpes simplex virus infections in the outpatients of Obstetrics and Gynecology Clinic. One hundred and two patients (mean age;35 years) were included in the study. None of the patients had clinical manifestations of Herpes simplex virus .The samples were collected from cervix by using sterile swabs and transported to the laboratory. Following the extraction of the samples, a PCR method was used for the detection of Herpes simplex virus DNA and 12 of 102 patients (12%) were found to be positive

As a result of our study, it is clear that Herpes simplex virus can be detected by PCR from the smears taken routinly from the symptom free outpatients of Gynecology and Obstetrics Clinics.

3-23

USING DIGENE HPV SPECIMENS FOR *C.TRACHOMATIS* AND HSV-2 POLYMERASE CHAIN REACTION

Öktem İMA, Sayıner AA, Kırdar S, Abacıoğlu YH
Dokuz Eylül University, School of Medicine,
Department of Microbiology and Clinical Microbiology, İzmir, TURKEY

Objective:To evaluate the appropriateness of the endocervical swab samples collected with Digene Specimen Transport Medium (HPV DNA Digene Hybrid Capture System, USA) for *C.trachomatis* and HSV-2 DNA PCR before and after the denaturation step of the HPV DNA hybridization test.
Method:Endocervical specimens were collected from four patients and divided into two aliquots (A, B). First aliquots (A) were spiked with *C.trachomatis* and HSV-2 positive culture cells. Spiked and non spiked specimens were again divided into two aliquots (A1, A2, B1, B2). DNA content of the aliquots A1 and B1 were extracted by a special kit (Marcherey Nagel, Germany). The aliquotes A2 and B2 were denaturated with NaOH and heat as the kit required for HPV-DNA hybridization assay. Samples were tested for HPV-DNA hybridization assay, *C.trachomatis* PCR and HSV-2 PCR. Denaturated samples were purified by using sephadex columns before being used for PCR assays.
Results:

ASSAY	SAMPLES*			
	A (spiked)		B	
	A1 (DNAext)	A2(denaturated)	B1(DNAext)	B2(denaturated)
HPV DNA	2/2	2/4	ND	2/4
C.trachomatis PCR	4/4	4/4	0/4	ND
HSV-2 PCR	4/4	4/4	0/4	ND

* Number of the positive samples / number of the tested samples, ND: not done
Conclusion: Endocervical swab samples collected in Digene Transport Medium were successfully used for *C.trachomatis* and HSV-2 PCR after DNA extraction or NaOH denaturation. Samples that were denaturated with NaOH required purification by sephadex columns to remove the excess bases from the medium.

3-24

CLONING AND EXPRESSION OF THE MATRIX GENE OF RINDERPEST VIRUS (RPV) VACCINE STRAIN

Azkur K[1], Bolat Y[1], Doymaz MZ[2]
University of Fırat, Faculty of Veterinary,
[1]Department of Virology,
Faculty of Medicine,
[2]Department of Microbiology and Clinical Microbiology, Elazığ, TURKEY

Rinderpest virus (RPV) is a major cause of mortality and morbidity in cattle. The aim of this study was the cloning and expression of the matrix gene of RPV in eucaryotic cells. For this purpose, Vero cells were infected with RPV RBOK vaccine strain. Total RNA isolation was performed and cDNA was obtained after reverse transcription (RT). Amplified PCR products were purified from agarose gel. The matrix gene,1047 bp in length, was cloned into Topo XL PCR cloning vector. PCR-screening and enzyme digestion assays were carried out to verify the presence of the matrix gene. Afterwards, the matrix gene cloned into the cloning vector was transferred into the pCI-neo mammalian expression vector using EcoR I and Sal I enzymes restriction sites. Recombinant pCI-neoMatrix was transfected into Vero cells. The presence of matrix proteins was showed by both SDS-PAGE and western immunoblotting.

3-25

MONOCLONAL ANTIBODY PRODUCTION AGAINST HEMAGGLUTININ PROTEIN OF RINDERPEST VIRUS AND IT'S CHARACTERIZATION

Gülaçtı İ, Bulut H, Bolat Y
University of Fırat, Faculty of Veterinary,
[1]Department of Virology, Elazığ, TURKEY

In this study, monoclonal antibody (MAk) production against hemagglutinin protein of Rinderpest virus (RPV) and its characterization were reported. For MAk production, RBOK vaccinia strain of RPV was grown in VERO cells. Then, splenocytes from Balb/c mice immunized with RPV-RBOK and myeloma cell line (FO) were fused by polyethylene glycol (PEG). For the selection of hybrid cell lines, the cell culture media containing hypoxanthine-aminopterin-thymidine (HAT) was used. An ELISA was carried out to select hybrid cell line producing antibodies against H protein. Western immunoblot method and neutralization test were used for characterization of anti-H monoclonal antibody.

3-26

THE USE OF RECOMBINANT HEMAGGLUTININE (rH) PROTEIN OF RINDERPESTVIRUS (RPV) IN ENZYME IMMUNOASSAY (EIA)

Bulut H[1], Bolat Y[1], Bulut Y[2], Özdarendeli A[2], Doymaz MZ[2]
University of Fırat, Faculty of Veterinary,
[1]Department of Virology,
Faculty of Medicine,
[2]Department of Microbiology and Clinical Microbiology, Elazığ, TURKEY

In this study, RPV recombinant hemagglutinine protein fused with A region of *Staphylococcus aureus* was expressed in *Escheria coli* and purified by IgG affinity chromatoraphy. rH protein was also used to establish enzyme immunoassay. Therefore, to prevent binding of Ig G to the protein A the wells coated with the rH proteins were blocked by human serum. Afterwards, RPV antigens were added to the wells to evaluate this assay. For this aim, serum of the mice immunized with RPV was used for EIA. Our data indicated that the rH protein fused with protein A of *Staphylococcus aureus* can be used reliably for enzyme immunoasay.

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SPONSORS

GENOMED

DIOMED

METİS

MERCK SHARP DOHME

BIOMERIEUX

MEDTEK

FARMA

GENOMAR

MOLLERGEN

DIOMED 'LE HEPSİNE ULAŞABİLİRSİNİZ

MAST ÜRÜN GRUBU

- Disk Difüzyon Duyarlılık Testleri
- Antibiyotik Duyarlılık Testleri
- Mastscanellte Sistem (Cihaz)
- Matring-S
- Methicillin Stripleri

- Multipoint Teknoloji
- Adatab Tabletler
- Mast Id

- Kültür Vasatları ve Selektif Supplementler
- Dehidreler
- Selektif

- Bakteriyolojik Aglütinasyon Antiserumları
- Salmonella
- Shigella
- V. Cholera
- E. Coli

- İdentifikasyon Ürünleri
- Mast Id Campylobacter İdentifikasyon Sistemleri
- Mast Id Campylobacter Biotyping Test
- Mast Rapid Slide Latex Test
- İdentifikasyon Stripleri ve Mastringler
- İdentifikasyon Diskleri
- Mrsa İdentifikasyonu
- Candida Spp. İdentifikasyonu

- Genel Laboratuvar Ürünleri
- Bakteriüri Test Stripleri
- Mast Cryobank
- Mast Demodishes
- Mast Qc Stif Saf Suşlar

- İmmunodiagnostik Testler
- Syphilıı (EIA)
- Clamydia (EIA)
- Cmv (EIA)
- Salmonella (EIA)
- Neospora (EIA)
- Anti-Tpo (EIA)
- Obstetrik Ürünleri
- Gizli Kantesti

DİOMED ÜRÜN GRUBU

Üretimini Yaptığımız Ürünler

- Dio-Pet: Disposable Petri Kutusu
- Dio-Media: Hazır Besiyeri
- Dio-Swab: Plastik Pamuklu Ekövyon Çubuk
- Dio-Transportswab: Vasatlı Steril Taşıma Tüpü
- Dio-Bacit: Beta Hemolitik Tarama Besiyeri
- Dio-Helico: Helicobacter Pylori Hızlı Tanı Kiti
- Dio-Safeproses: Balgam ve Sıvı Örnek Kontaminasyon Dekontaminasyon Kiti
- Dio-Sensitest: Hızlı Antibiogram Teşhis Kiti
- Dio-Sensimedia: Hızlı Antibiogram Teşhis Kiti
- Dio-Hemoculture: Bifazik Kan Kültür Sistemi
- Dio-Membrane: Mikrobiyolojik Filtrasyon Analiz Sistem
- Dio-Slide: Bakteri Kontaminasyonu için Transport Sistemleri
- Dio-Gas: Kazn Gazı Numune Alıcı Test
- Dio-Spatül: Plastik Dil Baskısı
- Dio-Edta K3: K3 Edtalı Tüp
- Dio-Plate: Mikrotiter Steril
- Dio-Plastüp: 12x56 Kapaklı Test Cam Tüpü P.P.
- Dio-Rack: 16 Ml Tüp için Otoklava Dayanıklı Sistemler
- Dio-Cup: Steril, Non Steril İdrar Kapları
- Dio-Tüp: Vakumlu Kan Alma Tüpleri

Satışını Yaptığımız Ürünler

- Laboratuvar Cam Malzemeleri
- Analitik Saflıkta Laboratuvar Kimyasalları
- Ar-Ge, An Aliz Saflıkta Kimyasal Maddeler
- Teknik Saflıkta Kimyasal Maddeler
- Tıbbi Ve Laboratuvar Sarf Malzemeleri
- Cihaz Kit Ve Sarf Malzemeleri
- Laboratuvar Cihazları
- Dio-Scrub: Dezenfektanlı El Temizleme Fırçası
- Dio-Care: Dezenfektanlı Vücut Fırçası

HENKEL ECOLAB HASTANE HİJYENİK ÜRÜNLERİ

- Incidur: Yer Ve Yüzey Dezenfektanı
- Incidur Spray: Yüzey Dezenfektanı
- Minudes: Yer Ve Yüzey Dezenfektanı
- Sekusept Pulver: Alet Dezenfektanı (Toz)
- Sekucid-N: Soğuk Sterilizasyon Maddesi
- Spitacid: El Antiseptisi
- Manipür: Dezenfektanlı Sıvı Sabun
- Secumatic Fnp: Alet Dezenfektanı (Sıvı)
- Secumatic Fre: Pas Çözücü
- Skinsept Mucosa: Sümüksel Zar Antiseptisi
- Secumatic İs: Cerrahi Alet Yağlayıcı
- Sekudrill: Döner Başlıklı Aaletler için Dezenfekltan Solüsyon
- Peresal: Hemodiyaliz Alet ve Ekipmanları Dezenfektanı
- Incides Mendi Yedek: Küçük Alet ve Yüzeyler için Hızlı Dezenfektan
- Incides Mendil: Küçük Alet ve Yüzeyler için Hızlı Dezenfektan
- Dozaj Pompası: Incidur Sozaj Pompası 20 Ml
- Druckpump: 1 Lt Hacimli Incudur Püskürtücü
- Henkel DG-1: Elektronik Dozaj Pompası

Sanayi Cad. No:19 Dudullu, 81260, İstanbul - Türkiye • Tel: 0 216 420 03 73 (6 Hat) • Fax: 0 216 466 63 01
E-mail: diomed@diomed.com.tr, diokalite@diomed.com.tr
www.diomed.com.tr