

Programme and Abstracts Book

Seventh International Meeting of the European Laboratory Working Group on Diphtheria

Vienna, Austria

12-14 June 2002



Seventh International Meeting of the European Laboratory Working Group on Diphtheria

European Laboratory Working Group on Diphtheria: ELWGD

Established 1993

and

Diphtheria Surveillance Network: DIPNET

Established 2002

*'A collaborative and co-ordinated approach to the
epidemiology and microbiology of diphtheria'*

First International Meeting

PHLS Central Public Health Laboratory, London, United Kingdom
April 1994

Second International Meeting

KTL National Public Health Institute, Helsinki, Finland
June 1995

Third International Meeting

Institut Pasteur, Paris, France
June 1996

Fourth International Meeting

Cantacuzino Institute, Bucharest, Romania
June 1997

Fifth International Meeting

AHEPA University Hospital, Thessaloniki, Greece
June 1998

Sixth International Meeting

European Commission, Brussels, Belgium
June 2000

Seventh International Meeting

University of Vienna, Vienna, Austria
June 2002

*Seventh International Meeting of the
European Laboratory Working Group on Diphtheria*

Vienna, Austria, 12-14 June 2002

Scientific Programme and Abstracts

Meeting organised by:

WHO Collaborating Centre for Diphtheria and Streptococcal Infections
Respiratory and Systemic Infection Laboratory
PHLS Central Public Health Laboratory, London, UK

Department of Traumatology
University of Vienna, Vienna, Austria

Federal Ministry of Social Security and Generations, Vienna, Austria

Vienna Medical Academy, Vienna, Austria

WHO Regional Office for Europe, Copenhagen, Denmark

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Local Organising Committee

Alexander Kolonja, Austria
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Helga Donhauser, Austria
Romana Koenig, Austria

>Welcome

On behalf of the Organising Committee, I would like to extend my very warm personal greetings to you all. It is a special pleasure to welcome you to the Seventh International Meeting of the European Laboratory Working Group on Diphtheria in the beautiful city of Vienna. This is a 'landmark meeting' in that it brings together for the first time not only the key microbiologists but the key epidemiologists globally that are responsible for diphtheria in their own respective countries.

We welcome colleagues from 40 different countries: 12 EU member states, six EU associated countries, 12 countries of the Newly Independent States (NIS) of the former Soviet Union, in addition to Norway, Switzerland, Israel, Turkey, India, Argentina, Brazil, Canada, USA, and Vietnam. In particular, we welcome participants from Argentina, India, the Czech Republic, Estonia, Germany, Luxembourg, the Netherlands, and Spain who are attending the meeting for the first time.

ELWGD was established by WHO EURO in July 1993, with only nine participating countries, and I would like to thank those 'founder members' that are still within our network: Izabella Mazurova, Galina Tseneva, Tatiana Glushkevich, Patrick Grimont, and Jaana Vuopio-Varkila for their support, contributions and friendship.

My thanks also to all colleagues who have joined the ELWGD since 1993 and, in particular, we very much welcome our epidemiology colleagues to this diphtheria arena! Therefore, it seems appropriate to propose that the network should be re-named 'The Diphtheria Surveillance Network: DIPNET' which will encompass both epidemiological and microbiological (ELWGD) aspects of diphtheria and other infections caused by potentially toxigenic corynebacteria.

The diphtheria international network has grown from strength to strength and is reflected by the many achievements and publications within the fields of the microbiology and epidemiology of diphtheria thanks to WHO EURO, the European Commission, and other agencies and organisations that have supported the activities during the last eight years.

During the last few years there have been many pivotal developments and this conference is the only forum for the documentation of such achievements. The key facts are that:

- New epidemics are emerging in different parts of the world and imported cases from epidemic areas to European and other countries are still occurring.
- Atypical and unusual manifestations of disease caused by other potentially toxigenic corynebacteria are also being documented.
- The genome sequence of the organism *Corynebacterium diphtheriae* has now been completed by the Sanger Centre.

My special gratitude goes, as always, to the dedicated and enthusiastic diphtheria 'teams' at the PHLS: Aruni De Zoysa, Gina Mann, Carole Kelly, and Ana Da Costa; and to the PHLS 'out of hours diphtheria team': Gina Mann, Frances Knight, Tony McNiff and Nita Doshi for their hard work. My special thanks to the 'honorary' member of our team, Roman Kozlov, for his hard work and efforts over the years.

Also special thanks to my colleagues within the PHLS Communicable Disease Surveillance Centre, in particular, Natasha Crowcroft, Joanne White and Anne-Claire de Benoist, for their support, advice, and collaboration.

My gratitude as always to the Public Health Laboratory Service, in particular to our Director, Robert George, for his support and invaluable advice and also to the Director of the Central Public Health Laboratory, Peter Borriello, for constant support of all our diphtheria activities and programmes.

Also, the continuous support of our WHO colleagues deserves a special mention, in particular to Nedret Emiroglu, and we look forward to our future collaborations with WHO EURO.

Our gratitude to the European Commission for support of our EC funded diphtheria programmes, BioMed 2, BMH4.98-3793, INCO Copernicus IC15.CT98-0302, and, more recently, DG SANCO 122/SID/2001.

Lastly, but by no means least, our gratitude and appreciation to our hosts and organisers, Stefan Marlovits, Alexander Kolonja, Karin Stegg, and Helga Donhauser, for their precious time and efforts in organising this meeting in the beautiful city of Vienna – many thanks for your enthusiasm, hard work and hospitality!

During the next few days we will again have the opportunity to share with each other all the exciting developments that have occurred since we last met in June 2000. I wish you all a successful and fruitful conference and an equally enjoyable stay in Vienna.



Androulla Efstratiou
ELWGD/DIPNET Co-ordinator
June 2002

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EC DG RTD INCO Copernicus IC15CT98-0302 programmes

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**Seventh International Meeting of the
European Laboratory Working Group on Diphtheria**

Vienna, Austria, 12-14 June 2002

Conference Venue: General Hospital Vienna
Waehringer Guertel 18-20
A-1090 Vienna

Day 1 Wednesday 12 June 2002

12.15 – 13.15

Registration

13.15 – 14.05

Welcome Addresses

Federal Ministry of Social Security and Generations

Dr Herbert Haupt

**Town-Councillor for Public Health and Medicine of the City
of Vienna**

Dr Elisabeth Pittermann-Höcker

Medical Director of the General Hospital of Vienna

Prof Reinhard Krepler

**Head of the Department of Traumatology of the University
of Vienna**

Prof Vilmos Vécsei

ELWGD Hosts

Dr Stefan Marlovits, Austria

Dr Alexander Kolonja, Austria

Dr Karin Stengg, Austria

ELWGD/DIPNET Co-ordinator

Dr Androulla Efstratiou, United Kingdom

Regional Adviser, WHO EURO

Dr Nedret Emiroglu

ELWGD NIS Representative

Dr Izabella Mazurova, Russia

Session 1

European Commission and WHO Programmes on Diphtheria and Other Infectious Diseases

Chairpersons: Dr N Emiroglu and Dr L Serafini

14.05 – 14.30	Diphtheria in the European Region of WHO <u>N Emiroglu</u> (A1.1)
14.30 – 14.50	EUVAC-NET: creation and operation of a surveillance community network for vaccine preventable infectious diseases <u>S Glismann</u> (A 1.2)
14.50 – 15.10	The IRIDE Network: information for action in Europe <u>S Salmaso</u> (A 1.3)
15.10 – 15.30	European Commission Sixth Framework Programme: 2002 to 2006 <u>L Serafini</u> (A 1.4)
15.30 – 16.00	BREAK
16.00 – 16.20	Impact of European Commission (EC) DGRTD, BioMed 2, INCO-Copernicus and EC DG SANCO programmes on diphtheria surveillance: ELWGD and DIPNET <u>A Efstratiou</u> , R Kozlov, S Lai, P A D Grimont and partners of the EC Diphtheria programmes (A 1.5)
16.20 – 16.40	EU and global assessment of microbiological diagnostics for diphtheria <u>C Kelly</u> , A Efstratiou, S Lai, R Kozlov, Partners of the EC DG SANCO DIPNET programme and the ELWGD (A 1.6)
16.40 – 17.10	Evaluating European surveillance of diphtheria for the new DG SANCO DIPNET programme <u>A C de Benoist</u> , J White, A Efstratiou, C Kelly, N Crowcroft (A 1.7)
17.10 – 17.30	General Discussion
19.30	Official Reception at the City Hall by the Mayor of Vienna

Session 2

Epidemiology of Diphtheria In Europe and Beyond

Day 2 Thursday 13 June 2002

Chairpersons: Dr A Efstratiou and Dr S Salmaso

09.00 – 09.15	<i>Corynebacterium diphtheriae</i> isolations in Finland: 1993 to 2002 <u>J Vuopio-Varkila</u> , S Salmenlinna, A Soininen, P Nuorti (A 2.1)
09.15 – 09.30	Toxigenic isolates of corynebacteria in the United Kingdom <u>J M White</u> , G Mann, N Crowcroft, R C George, A De Zoysa, C Kelly, A Efstratiou (A 2.2)
09.30 – 09.45	<i>Corynebacterium diphtheriae</i> surveillance in Belgium <u>D Piérard</u> , L Eeckhout, D Stevens, S Lauwers (A 2.3)
09.45 – 10.00	<i>Corynebacterium diphtheriae</i> carriage amongst children of Athens, Greece H Alexandrou-Athanassoulli, M Revena, K Lytina, I Pavlopoulou, <u>A Pangalis</u> (A 2.4)
10.00 – 10.15	Epidemiology of diphtheria in the Czech Republic <u>B Kriz</u> (A 2.5)
10.15 – 10.45	BREAK
10.45 – 11.00	Surveillance of diphtheria in Estonia <u>U Jöks</u> (A 2.6)
11.00 – 11.15	Organisation of nationwide surveillance of diphtheria in Slovenia <u>H Ribič</u> , A Kraigher, and members of the collaborating group (A 2.7)
11.15 – 11.30	Diphtheria status in India and data from a sentinel centre in Delhi, India <u>N C Sharma</u> , K N Tiwari, R C Panda, R Dhillon (A 2.8)
11.30 – 11.45	Current clinical and epidemiological aspects of <i>Corynebacterium diphtheriae</i> infections in Argentina <u>N A Leardini</u> , M A Prieto, C P Martinez, L A Aguerre (A 2.9)
11.45 – 12.00	Nasopharyngeal <i>Corynebacterium ulcerans</i> diphtheria in the Netherlands L G Visser, N Peek, E F Schippers, A van Dam, E Kuijper, C Swaan, <u>J F P Schellenkens</u> (A 2.10)
12.00 – 13.00	LUNCH

Session 3

Microbiological Surveillance of Diphtheria in the Newly Independent States (NIS) of the Former USSR

Chairpersons: Dr I Mazurova and Dr J Vuopio-Varkila

13.00 – 13.15	Laboratory diagnosis of diphtheria as part of diphtheria surveillance in Russia <u>I K Mazurova</u> , VG Melnikov, S Yu Kombarova, O Yu Borisova, E A Bugayev (A 3.1)
13.15 – 13.30	Levels of antitoxin antibodies and immunity to diphtheria amongst the populations of the northwestern district of the Russian Federation during the post-epidemic period: 1996 to 2001 <u>G Ya Tseneva</u> , U N Yakovleva, N I Akatova, V G Zhavoronkov, V S Yakovleva (A 3.2)
13.30 – 13.45	Epidemic of diphtheria in Latvia <u>A Griskevica</u> (A 3.3)
13.45 – 14.00	Microbiological surveillance of <i>Corynebacterium diphtheriae</i> in Latvia <u>I Selga</u> , I Drieska (A 3.4)
14.00 – 14.15	Current diphtheria situation in Smolensk region <u>R S Kozlov</u> , O I Sukhorukova, I P Golubeva, O V Senatorovav (A 3.5)
14.15 – 14.30	Results of microbiological monitoring for diphtheria in the Ukraine: 1992 to 2001 <u>T Glushkevich</u> (A 3.6)
14.30 – 15 00	BREAK

Session 3 (continued)

Microbiological Surveillance of Diphtheria in the Newly Independent States (NIS) of the Former USSR

Chairpersons: Prof H Kollaritsch and Prof G Tseneva

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| 15.00 – 15.15 | Modern conditions of laboratory diagnosis of diphtheria in Tajikistan and retrospective analysis of data in dynamic of epidemic process
<u>M Boltaeva</u> (A 3.7) |
| 15.15 – 15.30 | Diphtheria in the Republic of Georgia
<u>T Gomelauri</u> , M Kekelidze, M Gelenidze, N Tarkhasvili, E Jhorjholiani, N Jamaspishvili, P Imnadze (A 3.8) |
| 15.30 – 15.45 | Current situation with diphtheria in St Petersburg, Russia
<u>O Narvskaya</u> , E Timofeeva, E Limeschenko, L Loseva, I Mokrousov (A 3.9) |
| 15.45 – 16.00 | Microbiological control of diphtheria in the Republic of Moldova
<u>P Galetchi</u> (A 3.10) |
| 16.00 – 16.15 | Immunity to diphtheria from individuals from risk groups in the Kirov District of St Petersburg
<u>V N Antipov</u> , G Ya Tseneva, U N Yakovleva, N I Akatova, V V Dudareva (A 3.11) |
| 16.15 – 17.00 | General discussion on the NIS situation |
| 19.30 | Traditional Viennese evening at the Heuriger Fuhrgassl-Huber Restaurant |

Session 4

Clinical and Microbiological Aspects of Infection
Caused by Potentially Toxicogenic and Other *Corynebacteria*

Day 3 Friday 14 June 2002

Chairpersons: Dr N Crowcroft and Prof L Titov

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|---------------|--|
| 09.00 – 09.15 | Diphtheria antitoxin levels from three selected areas in Turkey
<u>B Levent</u> , D Kurtoglu, F D Ozkaya, A Gozalan, N Coplu, T Komiya, E Akbas, K Miyamura, B Esen and the Infectious Disease Control Project in Turkey Working Group (A 4.1) |
| 09.15 – 09.30 | Levels of anti-pertussis antibodies in comparison to diphtheria antitoxin levels in the Greek population
<u>A Tsakris</u> , A Polyzou, U Dafni, S Pournaras, S Patrinos, D Sofianou (A 4.2) |
| 09.30 – 09.45 | Seroprevalence of diphtheria immunity among injured adults in Austria
<u>S Marlovits</u> , R Stocker, A Efstratiou, K Broughton, A Kaider, V Vécsei, G Wiedermann, H Kollaritsch (A 4.3) |
| 09.45 – 10.00 | Penicillin tolerance of non-toxicogenic <i>Corynebacterium diphtheriae</i> strains isolated from cases of pharyngitis
<u>C von Hunolstein</u> , F Scopetti, A Efstratiou, K Engler (A 4.4) |
| 10.00 – 10.15 | Antibiotic resistant <i>Corynebacterium diphtheriae</i> strains circulating in Russia
<u>O Yu Borisova</u> , V G Melnikov, S Yu Kombarova, I K Mazurova (A 4.5) |
| 10.15 – 10.30 | Susceptibility to antibiotics of some <i>Corynebacterium diphtheriae</i> strains isolated in the Newly Independent States (NIS)
<u>A Diaconescu</u> , M Damian, B Marin, C Andronescu, L Titov, I Selga, A Melnic, G Tseneva (A 4.6) |
| 10.30 – 11.00 | BREAK |

Session 4 (continued)

Clinical and Microbiological Aspects of Infection
Caused by Potentially Toxicogenic and Other *Corynebacteria*

Chairpersons: Prof B Kriz and Dr S Marlovits

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| 11.00 – 11.15 | The characteristics of some biological properties in <i>Corynebacterium diphtheriae</i>
<u>K Iskhakova</u> , G Musaeva, N Shadmanova (A 4.7) |
| 11.15 – 11.30 | Use of cell wall antigens of non-toxicogenic <i>Corynebacterium diphtheriae</i> in an ELISA to determine diphtheria antibodies in human sera
<u>E A Shmeleva</u> , S I Makarova, T N Batalova, M P Korzhenkova, I G Baturina (A 4.8) |
| 11.30 – 11.45 | Invasion of cultured human epithelial cells by <i>Corynebacterium diphtheriae</i>
L Bertuccini, L Baldassarri, <u>C Von Hunolstein</u> (A 4.9) |
| 11.45 – 12.00 | Patterns of adherence to Hep-2 cells and ability to induce actin polymerization by toxigenic <i>Corynebacterium diphtheriae</i> strains
<u>A L Mattos-Guaraldi</u> , L C D Formiga, A F B Andrade Jr (A 4.10) |
| 12.00 – 12.15 | Non-toxicogenic corynebacteria associated with human infection: emerging pathogens
<u>N A Leardini</u> , M A Prieto, C P Martinez, L A Aguerre (A 4.11) |
| 12.15 – 13.15 | LUNCH |

Session 5

Molecular and Genetic Characteristics of *Corynebacteria* Symposium Sponsored by The Wellcome Trust

Chairpersons: Prof R K Holmes and Dr J Parkhill

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|---------------|---|
| 13.15 – 13.45 | The <i>Corynebacterium diphtheriae</i> strain NCTC 13129 genome project
<u>A M Cerdeño</u> , J Parkhill, and The Pathogen Sequencing Unit (A 5.1) |
| 13.45 – 14.00 | Whole genome visualisation, analysis, and comparison using Artemis and ACT
<u>J Parkhill</u> (A 5.2) |
| 14.00 – 14.15 | Toxigenic and non-toxigenic <i>Corynebacterium diphtheriae</i> in Turkey: characterisation of strains by using biological and molecular typing methods
<u>E Akbas</u> , N Coplu, B Levent, J Yatsuyanagi, S Nar, H Nakao, K Sovuksu, T Komiya, R Kayali, C Sonmez, B Esen (A 5.3) |
| 14.15 – 14.30 | Genotype characteristics of <i>Corynebacterium diphtheriae</i> strains in a period of low diphtheria incidence in Russia: 1997 to 2001
<u>S Yu Kombarova</u> , V G Melnikov, OYu Borisova, T V Platonova, I K Mazurova (A 5.4) |
| 14.30 – 14.45 | Etiological value of non-toxigenic <i>tox</i>-bearing (NTTB) <i>Corynebacterium diphtheriae</i> strains
<u>V G Melnikov</u> , S Yu Kombarova, O Yu Borisova, I K Mazurova (A 5.5) |
| 14.45 – 15.00 | Interstrain heterogeneity of non-toxigenic <i>tox</i> bearing (NTTB) <i>Corynebacterium diphtheriae</i>
<u>S A Gabrielyan</u> (A 5.6) |
| 15.00 – 15.30 | General discussion on ‘the way forward’ with the <i>Corynebacterium diphtheriae</i> genome sequence data |
| 15.30 – 16.00 | BREAK |

Session 5 (continued)

Molecular and Genetic Characteristics of *Corynebacteria* Symposium Sponsored by The Wellcome Trust

Chairpersons: Prof P Grimont and Dr T Popovic

- 16.00 – 16.20 **Use of transposon mutagenesis to analyse regulation and function of the diphtheria toxin repressor in *Corynebacterium diphtheriae***
D Marra Oram, A Avdalovic, [R K Holmes](#) (A 5.7)
- 16.20 – 16.35 **Adaptation of the international *Corynebacterium diphtheriae* ribotypes database to the RiboPrinter**
F Grimont, M Lejay-Collin, [P A D Grimont](#) (A 5.8)
- 16.35 – 16.50 **Molecular characterisation of clinical isolates of *Corynebacterium diphtheriae* isolated from northern states of India including Delhi**
[S S Thurkral](#), S Ahmad, N C Sharma (A 5.9)
- 16.50 – 17.05 **Molecular characterisation of non-toxigenic *tox*-gene bearing corynebacteria strains**
[M Damian](#), C R Usein, B Luca, M Florea, A Diaconescu (A 5.10)
- 17.05 – 17.15 **Molecular epidemiology of *Corynebacterium diphtheriae* and *C. ulcerans* strains isolated in Italy**
[C von Hunolstein](#), G Alfarone, F Scopetti, M Pataracchia, A De Zoysa, A Efstratiou (A 5.11)
- 17.15 – 17.30 **Characterisation of toxigenic *Corynebacterium diphtheriae* strains isolated in the UK from human and domestic cats**
[A De Zoysa](#), N Fry, D Taylor, W Reilly, RC George, N Crowcroft, J White (A 5.12)
- 17.30 – 17.45 **Travellers as human reservoirs of *Corynebacterium diphtheriae* – a story between England and Israel**
[E Marva](#), L Giladi, B Schnaidman, V Agmon, E Tallen-Gozani, R McCann, N Crowcroft, J White, A De Zoysa, A Efstratiou (A 5.13)
- 17.45 – 18.00 **Molecular-biological monitoring of *Corynebacterium diphtheriae* circulation in Belarus**
[V Kolodkina](#), L Titov, A Blizniuk, T Denisevich, P Grimont, S Lai, A Efstratiou (A 5.14)
- 18.00 – 18.15 **Closing Remarks**
A Efstratiou, N Emiroglu, I Mazurova, S Marlovits
- 20.00 **Farewell dinner at the Restaurant Stadtbeisl**

End of the scientific conference

SESSION 1

European Commission and WHO
programmes on
diphtheria and other infectious
diseases

A 1.1: Diphtheria in the European Region of WHO

N Emiroglu

Division of Technical Support and Strategic Development, Communicable Disease Control, Prevention, and Eradication, World Health Organization, Regional Office for Europe, Copenhagen, Denmark

The epidemic of diphtheria in the Newly Independent States (NIS) began in the Russian Federation in 1990 and effected all 15 NIS by the end of 1994. In 1990, the number of reported diphtheria cases in the NIS was 1,436. Cases increased to 19,604 in 1993 and then to 47,869 in 1994, and finally reached a peak of 50,434 in 1995. In 1995, cases in the NIS accounted for 88% of worldwide reported diphtheria cases.

Diphtheria control efforts started in the Russian Federation in 1992, with mass immunisation campaigns covering the majority of adults between 1992 and 1994. Control efforts and mass immunization campaigns in other NIS followed, achieving high coverage (80% and higher in all age groups) relatively quickly through mass immunisation. All these countries are now using high-potency vaccines in the primary series and have lower rates of childhood contraindications.

As a result of vigorous action taken in the Russian Federation and of collaboration between the diphtheria epidemic countries and the Interagency Immunization Coordination Committee (IICC), the incidence of diphtheria started declining in the Russian Federation in 1995 and in the other NIS in 1996. Furthermore, the decreasing trend in the region has continued in 1997, with a total of 7,182 cases (a decrease of 64%), and in 1998, 2,783 cases (a decrease of 62%). The number of cases reported to the European Region of WHO in 1999 and 2000 was approximately 1500.

In the beginning of the epidemic, the case fatality rate was very high, more than 20% in some countries, due to lack of antitoxins, improper case management, and delayed treatment. The age distribution of cases in this epidemic has been unusual as there has been a high proportion of cases among adolescents and adults. The geographic impact of the epidemic has largely been urban, following the geographic distribution of the population, except in the Central Asian Republics and the Caucasus where most of the population live in rural areas. At the present time, excellent progress in the control of diphtheria has been achieved in Armenia, Azerbaijan, Belarus, Estonia, Kazakhstan, Lithuania, Moldova, Turkmenistan, and Uzbekistan, where the incidence of the disease has reached low levels. The control of diphtheria still needs further improvement in Georgia, Kyrgyzia, Latvia, Russian Federation, Tajikistan and the Ukraine. Latvia is one of the countries with a high incidence rate and surveillance information is consistent with the usual epidemiology of the disease in a population with high levels of childhood vaccination, but incomplete adult coverage.

The European Laboratory Working Group on Diphtheria (ELWGD) was formed in 1993 with the participation of 20 countries from Western and Eastern Europe, the United States, Australia, and Southeast Asia. The ELWGD continues its collaborative and coordinated approach to support countries to improve diphtheria surveillance for early detection of cases and contacts by accurate microbiological surveillance and the establishment of a network of national and international laboratories.

The general plan for all countries is to focus on achieving high coverage through routine immunization, closing gaps in adult immunization, and proper management of diphtheria cases and contacts, including accurate diagnosis.

A 1.2: EUVAC-NET: creation and operation of a surveillance community network for vaccine preventable infectious diseases

S Glismann

Department of Epidemiology, Statens Serum Institut, Copenhagen, Denmark

In December 1999, an agreement was reached with the European Union (EU) (DG SANCO) whereby the Statens Serum Institut, Denmark (SSI) was to co-ordinate a collaborate project, the EUVAC-NET (Decision No. 2119/98 of the European Parliament and Council). The objective of the project was to create and operate a surveillance network of vaccine preventable infectious diseases with an emphasis on epidemiological and laboratory methods.

The two main diseases initially targeted were measles, coordinated by the SSI, and pertussis, undertaken by the Istituto Superiore di Sanità, Italy through an associated contract with the SSI. Participating countries included the fifteen European Union countries as well as Iceland, Norway and Switzerland. Gatekeepers representing each of the central surveillance institutions within each country were appointed who had knowledge of surveillance systems, vaccination programmes, and methods used for estimation of vaccination coverage. The first project period of 18 months consisted of a feasibility study with two questionnaire surveys to assess data resources at national level. The second project period of 15 months will be focusing on consolidating the network.

Action plans, case definitions, and recommendations were agreed upon with the established network of gatekeepers. Databases were created for both measles and pertussis surveillance and included historical data from most of the participating countries. Results from the two surveys were useful for defining the variables of minimal datasets. An assessment study for integration of the network into the Health Surveillance System on Communicable Diseases (HSSCD) was completed and a collaborative agreement (memo of understanding) was reached between the WHO EURO and the SSI.

It is feasible to create and operate a surveillance network for vaccine preventable disease. A close collaboration on technical issues among surveillance networks of vaccine preventable infectious diseases would be of mutual benefit. Managerial activities have taken longer than expected during this project, although the recently established Network Forum should facilitate the best use of managerial experience in the future. The EUVAC-NET could be a forum to facilitate this and objectives for the network may need to be redefined.

A 1.3: The IRIDE Network: information for action in Europe

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In 2000, the EU (DG SANCO) sponsored an inventory entitled IRIDE (Inventory of Resources for Infectious Diseases in Europe) aimed to provide reliable and timely information on the available resources for the control of the communicable diseases in the EU, as well as 15 additional countries that are candidates to join the EU in the future.

IRIDE is a European database that includes country-specific data organised in five major areas: statutory notification systems, non-statutory surveillance systems, outbreak management, institutions, and reference laboratories. Members of the European Charter of State Epidemiologists (CESE) were invited to act as national gatekeepers and were responsible for promoting and supervising data entry for their country. Counterparts from the new participating countries were also identified. Technical expertise was provided by CINECA (Interuniversity Consortium Information Management and Analysis Department, Bologna). A website (<http://iride.cineca.org>) was set up in October 2000 which allows participating countries to complete and update their questionnaires online. Access to the database on the website is divided into two parts: the public part, which is in the public domain, and the private part, which is password protected. Four reports that are automatically updated from the database are also available online and provide summary results of the major areas.

IRIDE provides a simple system for continuous updating of information relevant to the control of communicable diseases through a network of national gatekeepers each in charge of revising their specific part of the database.

A 1.4: European Commission Sixth Framework Programme: 2002 to 2006

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Following a political agreement made among the 15 Ministers of Research of the European Union on 10 December 2001 (common position of the Council), a new generation of European programmes of research is taking shape. The European Parliament and Council are expected to finalise their work on the Sixth Framework Programme (FP6) around the middle of 2002 and the new framework programme should start in early 2003. FP6 is focussed on the realisation of the European research area (ERA) whose main objective is to promote the integration of European research capacity and effort through the implementation of coherent and focussed approaches. This will be realised with the support of new instruments, designed to provide an integrating effect, like networks of excellence, integrated projects, and joint initiatives with member states (Article 169), but allowing, in the meantime, the continuity of certain classical actions used in the previous research programmes. It also intends to provide a structuring effect to horizontal policies (human resources, SMEs, infrastructures...) associating them to regional and national efforts and to other European initiatives. Finally, it will promote the co-ordination of national programmes with the objective of assuring synergy and coherence of European efforts of research.

The structure of this new programme, with a budget of 17.5 billion euros, is articulated around three big axes of action: integrating European research, structuring the ERA, and strengthening the foundations of ERA. The integration of European research is performed through seven priority themes, representing around three-quarters of its global budget.

Among those themes, there are three of particular importance for health: theme 1, "genomics and biotechnology for health" which covers research on advanced genomics and its applications for health and research on combating major diseases; theme 3, "nanotechnologies" which involves, among other topics, knowledge based multifunctional materials; and finally theme 5, "food quality and safety". Theme 1 is the most relevant for this audience and will focus on fundamental knowledge of functional genomics in all organisms and applications of knowledge and technologies in the field of genomics and biotechnology for health. This involves on one hand, gene expression and proteomics, structural genomics, comparative genomics and population genetics bioinformatics, and multidisciplinary functional genomic approaches to basic biological processes, and on the other hand technological platforms for development in the fields of new diagnostic, prevention, and therapeutic tools.

The focus of combating major diseases is on application-oriented genomic approaches to medical knowledge and technologies. Here the research effort will concentrate on combating cardiovascular diseases, diabetes, and rare diseases, combating resistance to antibiotics and other drugs, studying the

brain and combating diseases of the nervous system, studying human development and the ageing process. Additional effort will be placed on combating cancer and on confronting the major communicable diseases linked to poverty.

A 1.5: Impact of European Commission (EC) DGRTD, BioMed 2, INCO-Copernicus and EC DG SANCO programmes on diphtheria surveillance: ELWGD and DIPNET

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The diphtheria epidemics of the 1990s within the Newly Independent States (NIS) of the former Soviet Union have major implications for Europe and its associated countries, where there are many immigrants from the NIS and Eastern Europe. Imported infections in visitors from Western Europe, as a consequence of increased travel and trade, have been reported from almost every European Union (EU) member state. The reasons for the epidemics include decreasing immunisation coverage amongst infants and children, large gaps in immunity in adults, delays in implementation of control measures, large population movements, and the lack of supplies for prevention and treatment in many affected countries. All these factors have raised important issues concerning appropriate public health and microbiological surveillance.

Initially, two EU funded diphtheria projects, Biomed 2 and INCO-Copernicus (1998 to 2002) focused upon collaborative and coordinated approaches to microbiological surveillance of diphtheria within and between European countries and beyond. As a result, links between the NIS and Western Europe have been significantly strengthened. International collaboration has been essential to monitor the spread of the disease, the pathogenesis of the organism, and to promote the necessary research and development within this field. Thus, the creation of a network of European Reference Centres represents not only a new initiative but is also a good example of coordinated activities leading to joint research collaborations. Exploitation at the public health level has led to significant improvements and innovations in microbiological surveillance and diagnosis, for example harmonisation of methodologies between countries for laboratory diagnostics, development of novel tests for laboratory diagnosis, establishment of external quality assurance

schemes to monitor laboratory proficiency, and the establishment of a definitive genotype database for 'rapid tracking of epidemic strains'. In addition, training visits for NIS scientists, workshops, and symposia have been organised throughout Europe to maintain and increase awareness. Symposia were held in more than 15 countries, with multilingual presentations according to the main national language of that country (English, French, Italian, Greek, Finnish, Russian). This has been another unique approach. All these countries, in collaboration with WHO EURO and the European Laboratory Working Group on Diphtheria (ELWGD) with a total of 35 countries worldwide, have raised awareness of this disease.

The epidemics during the last 12 years remind us of the danger of infectious diseases such as diphtheria to populations; there is still much to be learned about the epidemiology of this disease. This area will be strengthened by the recent EC award from DG SANCO to undertake a feasibility study for diphtheria surveillance amongst all EU member states. The characteristics of the epidemics and the achievements from these programmes can be used to predict how future epidemics might spread and what epidemiological and microbiological control measures should be taken.

A 1.6: EU and global assessment of microbiological diagnostics for diphtheria

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One of the main objectives of the DG SANCO DIPNET project is to determine criteria for the standardisation and dissemination of protocols for the microbiological and epidemiological surveillance of diphtheria. This project will enable the evaluation and selection of optimal methods and typing strategies and support their dissemination to diphtheria reference centres throughout Europe and beyond. During the past few years, ELWGD questionnaires were sent to diphtheria reference centres throughout the world requesting details of their reference services. The questionnaires were completed by 28 countries, both within the EU such as Austria, Belgium, Denmark, Finland, France, Germany, Italy, Sweden, UK, and beyond such as Australia, Belarus, Canada, India, Israel, Kazakhstan, Kyrgyzia, Latvia, Moldova, Norway, Romania, Russia, Slovenia, Switzerland, Turkey, the Ukraine, USA, and Vietnam. Only 57% of the reference centres (16/28) completed their most recent questionnaire after 2000, so the facilities and services provided in the remaining centres may have changed since their questionnaires were returned.

Fifty-seven per cent of the centres (16/28) that received the questionnaire were National Diphtheria Reference Centres for their country, although a further 14% (4/28) had applied for designation at the time the forms were completed. Three-quarters of the centres (21/28) provided a reference service for their entire country and 29% (8/28) also provided a services for cultures referred from other countries. Most countries (85%, 24/28) had a national communicable disease surveillance or epidemiology system responsible for diphtheria surveillance, although only 71% (20/28) said that their laboratory records were computerised or easily available. The number of isolates received, the number of diphtheria cases identified, and the proportion that were associated with travel all varied greatly between countries. Only 32% of the countries questioned routinely screened throat swabs for the presence of *Corynebacterium diphtheriae*.

Standardisation of methodologies for reference laboratories is essential, especially for toxigenicity testing, serological testing, and molecular typing. Information regarding the current methodologies for biotyping and identification of *C. diphtheriae* including primary selective media, screening tests, and biotyping, toxigenicity testing, and antibiotic sensitivity testing were recorded in the questionnaire. Details were also requested about the source and types of serum, antitoxin, controls, PCR primer sets etc. Twenty-nine per cent of reference centres (8/28) said that they experienced problems obtaining culture media. There was some variability in the methodologies and control strains used for biotyping tests and the Elek test. Thirty-nine per cent of the centres (11/28) performed epidemiological typing and the most common method used was ribotyping. Sixty-one per cent (17/28) confirmed that they had collections of isolates from outbreaks and systemic cases that they were willing to exchange with other reference centres. Seventy-one per cent of the centres (20/28) performed serological testing for diphtheria antitoxin levels using a variety of methods, however the limits of normality were not consistent. Updated information from all centres, particularly EU member states, is essential to ascertain the level of standardisation within diphtheria diagnostic laboratories.

A 1.7: Evaluating European surveillance of diphtheria for the new DG SANCO DIPNET programme

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Standards for microbiology and public health surveillance of diphtheria and other diseases caused by corynebacteria in Europe do not exist, despite ongoing epidemics in the European region.

Practices vary considerably across EU member states, therefore it is difficult to ascertain whether existing systems are adequately prepared for the detection of new cases. Prompt identification is essential for the early treatment of cases, timely public health response, and monitoring of vaccination programmes.

Existing information on diphtheria surveillance in Europe was evaluated for the new DG SANCO DIPNET project. A pilot survey was carried out to explore diagnostic microbiology, clinical practice, and public health surveillance. The objective is to develop an approach for evaluating surveillance of diphtheria at a European level.

SESSION 2

Epidemiology of diphtheria in Europe and beyond

A 2.1: *Corynebacterium diphtheriae* isolates in Finland: 1993 to 2002

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In April 1993, the first imported case of diphtheria caused by toxigenic *Corynebacterium diphtheriae* var *gravis* was identified in Finland after nearly thirty years of no diphtheria cases being reported. Since then, a total of fifteen isolations of *C. diphtheriae* have been reported, the majority produced toxin (13/15) and were var *gravis* (10/15). Twelve of the cases had epidemiological links to Russia and were caused by toxigenic *C. diphtheriae*, one case of non-toxigenic *C. diphtheriae* var *mitis* was in a laboratory worker with tonsillitis, and, in the remaining two cases, the origin was unknown. Based on ribotype analysis, most of the var *gravis* isolates were indistinguishable from the Russian epidemic clone (D1/D4) while only one had a different pattern. The var *mitis* strains represent three different ribopatterns, two of which were similar to patterns found among strains isolated from Russia during the last decade. The clinical picture varied from asymptomatic carriage to severe tonsillitis with complications. Two of the cases were fatal. Three of the cases were children, but the majority were middle-aged men (8/15) or women (3/15). Only two of the cases were household contacts. Despite extensive traffic across the border, the small number of diphtheria cases identified in Finland in recent years suggests that the overall immunity of the population is relatively good. Adults should ensure, however, that they receive appropriate diphtheria booster vaccinations, especially when travelling to Russia or the Baltic states.

A 2.2: Toxigenic isolates of corynebacteria in the United Kingdom

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Since 1996, routine screening of throat swabs for potentially toxigenic corynebacteria has been recommended as a standard operating procedure (SOP) by the Public Health Laboratory Service (PHLS) in the United Kingdom (UK). The PHLS Streptococcus and Diphtheria Reference Unit identified 27 isolates of

toxigenic corynebacteria in the five-year period between 1997 and 2001 from throat swabs and other samples types referred for toxigenicity testing. A further four isolates have been identified during the first three months of 2002.

Only 11 isolates were *Corynebacterium diphtheriae* strains; eight *var mitis* and three *var gravis*. Nine were associated with travel (the Indian sub continent, Africa, the Baltic, and Israel), and included one 'classical diphtheria' and three cutaneous infections. Two cases had no history of travel, although one lived in a Bangladeshi community in the UK, and the other was a laboratory-acquired infection.

Two thirds (20/31) of all toxigenic isolates of corynebacteria were identified as *C. ulcerans*. Seventeen were identified from throat swabs, including two patients with classical pseudomembrane of the pharynx, one of whom died, and three cutaneous infections. Infection with *C. ulcerans* in humans is 'traditionally' associated with consumption of raw dairy products or contact with farm animals. No such risk factors were identified for about half of the cases, including the 'classic' cases.

Recent isolates of toxigenic *C. ulcerans* from domestic cats, as well as the possibility of person-to-person spread, highlights the importance of further research into the role of *C. ulcerans*. Surveillance of corynebacteria through laboratory screening of throat swabs must be maintained.

A 2.3: *Corynebacterium diphtheriae* surveillance in Belgium

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In Belgium, the Scientific Institute of Public Health monitors certain pathogenic agents such as *Corynebacterium diphtheriae* through a sentinel network of 127 laboratories representing 46% of all recognised private or hospital microbiology laboratories (<http://www.iph.fgov.be/epidemio/>). Participation is voluntary and unpaid. In addition, 38 reference laboratories are responsible for laboratory diagnostic testing for specific organisms, for confirmatory testing of samples from other laboratories, and for subtyping and antibiotic testing of isolates. It must be stressed that laboratories not participating in the sentinel network also have the opportunity to use these facilities.

In 1995, the Department of Microbiology of the AZ-VUB was designated as a reference laboratory for *C. diphtheriae*. Suspect isolates are identified, biotyped by biochemical tests, and toxigenicity testing is carried out by DNA amplification of the diphtheria toxin gene. Between 1995 and 2001, 14 isolates (0 to 5 per year, median: 2) were confirmed as *C. diphtheriae*, but all were found to be non-toxigenic. These isolates

were *var gravis* (6 isolates), *var belfanti* (4), *var mitis* (1), or were not biotyped (3). Although we have concluded that diphtheria is not present in our country, surveillance of this disease is still necessary. A recent study showed that at least 32% of the Flemish population has diphtheria antitoxin titres <0.01 IU/ml and are therefore susceptible to infection.

A 2.4: *Corynebacterium diphtheriae* carriage among children in Athens, Greece

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The recent epidemic of diphtheria in Russia and the Newly Independent States (NIS), as well as an increase in the number of immigrants from these countries to Western Europe, requires continuous surveillance of pathogenic corynebacteria to be performed.

Five hundred and fifty two children (511 of Greek nationality and 41 immigrants), a representative sample of the children in Athens attending nursery, elementary, and high schools, were screened for carriage of *C. diphtheriae* and *C. ulcerans*. The parents of these children completed questionnaires. Two hundred and five children (37%) were under 12 years and 349 (63%) were greater than 12 years (mean age: 10 (±4) years). Forty-nine per cent of the children were suffering from an upper respiratory tract infection and 20% were undergoing antibiotic therapy. Thirty-three per cent of the children came from homes where their parents were non-smokers and 57% where neither parent had had a university education. Throat swabs were inoculated onto Hoyle's tellurite and blood agar plates and, after an incubation period of 48 hours, any suspicious colonies were handled according to the procedures described in the European Region of WHO's *Manual for the Laboratory Diagnosis of Diphtheria*. Coryneforms were isolated in 20% of the samples but *C. diphtheriae* or *C. ulcerans* was not identified. Although carriage of pathogenic corynebacteria was not found among the children in Athens, the surveillance and screening for carriage should be continued.

A 2.5: Epidemiology of diphtheria in the Czech Republic

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Health care systems within the Czech lands (Bohemia, Moravia, and Silesia) of the late Austrian-Hungarian Empire and Czechoslovakia were comparatively well developed. Attempted measures to control epidemic spread of diseases can be traced back to the 16th Century. A truly functional reporting system for infectious disease was established in 1888 when the Ministry of the Interior issued a decree (No. 20,604 on 13th December 1888) which required infectious diseases, which included diphtheria and six other diseases, to be reported - "the municipal council be bound to report to the authorities the outbreak of infectious disease, meaning the first very case of diphtheria". *Report on Conditions of Health in the Czech Kingdom* reported the incidence of disease and was first published in 1892. The incidence of diphtheria in 1890 was reported to be 93.6 per 100,000. In 1894, the highest incidence was recorded (186.9 per 100,000) as well as the highest case fatality rate of 45.2%. The next peak in the secular trend of incidence rates was in 1915 (157.3 per 100,000) while the last was in 1943 (349.6 per 100,000). Compulsory vaccination was introduced in 1946 and DTP vaccine has been available since 1958. The indigenous transmission of diphtheria has not been reported since the mid 1970s. Serological surveys of antitoxin antibodies carried out since 1966 have revealed between 95-100% seropositives among the vaccinated population and 55% among non-vaccinated age groups. In the last survey in 1996, antitoxin levels of >0.01 IU/ml were detected in 97-100% of individuals aged between 1 to 19 years

A 2.6: Surveillance of diphtheria in Estonia

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Twenty years after the introduction of childhood vaccination in Estonia, the last case of diphtheria before a long diphtheria-free period was diagnosed in October 1964. The rate of vaccination among children stayed at approximately 90%, although the number of revaccinated children decreased. In 1977, a study was conducted which looked at the level of antitoxin antibodies and immunity to diphtheria in the adult population. The study showed low immunity in this group as antibodies were detected in only 55% of tested persons, with raised titres in only 31%. The testing of 2 to 5% of the population each year

monitored the spread of *Corynebacterium diphtheriae*, although no toxigenic strains were isolated between 1966 and 1977. Revaccination of 16 year old adolescents was introduced in 1983. The reappearance of toxigenic strains and the low immunity rate of the population justified the vaccination of population of 20 to 56 year olds between 1985 and 1987. A significant rise in the level of immunity within the population was observed, with antibodies found in between 74% and 94% of tested persons, and protective titres in 70%.

Despite all these implemented measures, however, diphtheria re-emerged in Estonia. In 1991, seven cases of diphtheria were reported including the death of an unvaccinated child. The incident rate was 0.4 per 100,000. Cases of diphtheria were reported in each of the following years except 1998 and 1999 and the incident rate rose from 0.4 to 1.3 per 100,000 in 1995. The re-emergence of diphtheria in Estonia was not due to local causes, but was part of an epidemic that began in Russia and its neighbouring countries. Imported cases occurred repeatedly in Estonia, but the spread of the disease was restrained with the use of effective preventative measures.

A 2.7: Organisation of the nationwide surveillance of diphtheria in Slovenia

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For over thirty years, Slovenia was free from diphtheria due to an immunisation coverage rate of over 95%. When a large epidemic of this disease spread across the Newly Independent States (NIS) in the 1990s, the risk of epidemic spread in Slovenia was unknown.

To clarify the risk of diphtheria in Slovenia, nationwide surveillance of diphtheria was begun in 1994. The results of a seroepidemiological study showed protective immunity in children and young adults, although there was evidence of a fall in the level of protective antibodies in adults. Between June 1994 and the end of 1999, a bacteriological study was carried out where 21,071 clinical samples from the upper respiratory tract were screened for *Corynebacterium diphtheriae*. The number of *C. diphtheriae* isolations was very low (one to seven isolates per year). All of the strains were non-toxigenic and most were *C. diphtheriae* var *belfanti*.

The central laboratory in Ljubljana coordinated the study and eight microbiological laboratories from all regions of Slovenia were included. A microbiologist from the central laboratory provided the algorithm for both the screening of potentially toxigenic corynebacteria and for the identification of suspicious isolates. All isolates of *C. diphtheriae* were sent to the central laboratory, where identification was confirmed and the Elek test was performed to determine toxigenicity. All confirmed strains have been frozen for

further analysis. The introduction of molecular techniques in Slovenia would not be cost effective as the rate of isolation of potential toxigenic corynebacteria is low. International collaboration of the central laboratory with other laboratories, including members of the ELWGD is essential.

A 2.8: Diphtheria status in India and data from a sentinel centre in Delhi, India

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Between 1990 and 1997, 32 states reported 56,091 cases of diphtheria to the government of India, with a fatality rate of 6%. During this time, a definite downward trend was observed. Thirteen of the states reported more than 1000 cases during this period, with West Bengal having reported the largest number of cases (32%), followed by Andhra Pradesh (23%). At present, the national communicable disease surveillance system for India is not carrying out surveillance of diphtheria, therefore the only available data from the Maharishi Valmiki Infectious Disease Hospital, Delhi can be described. One thousand, one hundred and ten suspected cases of diphtheria were admitted, 55% of whom were from Delhi alone, 31% from Uttar Pradesh, and 11% from Haryana. The male to female ratio ranged between 1.3:1 to 1.9:1. The largest number of cases were aged from 1 to 4 years (50%) followed by 4 to 9 years (28%). A seasonal trend was observed, as the number of isolations increased between July to November. Among both suspected and laboratory confirmed cases, the majority were unvaccinated (65% and 67%, respectively). The case fatality rate was higher amongst the confirmed cases, between 36% and 43%, compared to the rate for all cases (24% to 35%). Adult and adolescent cases, as well as clusters that occurred within families, were recorded during this period. Areas of endemic foci were identified within the city of Delhi and in adjoining states. Two outbreaks occurred during 2001, one in a specific region of Delhi and another in a village called Khekra. The proportion of cases that were laboratory confirmed increased from 3% in 1995 to 36% in 2001. Between 1999 and 2000, only 39 isolates were biotyped and tested for toxigenicity. Thirty-six of these isolates were *Corynebacterium diphtheriae* var *intermedius* (29 toxigenic and seven non-toxigenic), two were *C. diphtheriae* var *mitis* (both toxigenic), and one was *C. diphtheriae* var *gravis* (non-toxigenic). In the future, data will be presented on antibiotic sensitivity, which has recently been standardised, and methods are currently being evaluated.

A 2.9: Current clinical and epidemiological aspects of *Corynebacterium diphtheriae* infections in Argentina

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Epidemic diphtheria has not been reported in Argentina since 1990. As described in other parts of the world, the number and severity of cases declined in the last few decades. Nevertheless, a few sporadic cases of systemic infection produced by invasive strains of *Corynebacterium diphtheriae* have been observed. We describe three cases of endocarditis caused by *C. diphtheriae* that have been recently investigated. In the first case, *C. diphtheriae* was isolated from a blood culture from a paediatric patient without any previous underlying clinical condition. The second case was a six years old child who had congenital heart disease with a cardiac blow. After being hospitalised with a fever, *C. diphtheriae* var *intermedius* was isolated from three blood cultures. The third case was an alcoholic adult, with a prosthetic aortic valve. He was admitted to the hospital with a fever, a myocardial blow, and oedema of his right leg. *C. diphtheriae* var *gravis* was isolated from two blood cultures. All three cases were diagnosed as having been infected with non-toxigenic *C. diphtheriae*. These cases illustrate that *C. diphtheriae* may manifest severe invasive properties without the effects of diphtheria toxin. As sporadic cases with arterial embolism occur in completely immunised individuals, it is important that a correct diagnosis of endocarditis due to *C. diphtheriae* is made. Endocarditis caused by *C. diphtheriae* could become a public health problem as a large proportion of adults, both in developed and developing countries, do not have protective levels of immunity to *C. diphtheriae*. Furthermore, immunisation against *C. diphtheriae* can protect individuals from the toxic effects of *C. diphtheriae* but does not prevent against invasive disease. The pathogenic mechanisms of non-toxigenic strains remain unclear.

A 2.10: Nasopharyngeal *Corynebacterium ulcerans* diphtheria in the Netherlands

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A 59-year old woman was admitted to hospital after having a sore throat for three days and increasing dysphagia during treatment with oral penicillin for one day. The patient was afebrile, the soft palate and uvula were swollen, and a membranous exudate was seen on the soft palate and nasopharynx. There was no palpable cervical lymphadenopathy or soft-tissue swelling. She had not recently travelled abroad or been in contact with people who had recently travelled, and had not been vaccinated against diphtheria. The patient was barrier nursed, treated with intravenous penicillin, and fully recovered fully within four days. *Corynebacterium ulcerans* was isolated from a throat swab. The strain contained the diphtheria toxin gene, as shown by PCR with primers for the A and B portion of the gene. The Elek toxigenicity test was positive for the *C. ulcerans* strain. On admission, the patient's serum antibodies against diphtheria toxin, assessed in an internationally standardised TOBI assay, were undetectable (< 0.01 IU/ml) on admission and detectable (0.03 IU/ml) ten days after.

Two household contacts of the patient were screened, even though person-to-person transmission of *C. ulcerans* is rare, but *C. ulcerans* was not isolated. The patient had not recently been in contact with horses, cattle, or any other animals, except her domestic cat. She did not drink unpasteurised milk. The source of the infection remained unclear.

Although the last reported case of diphtheria in the Netherlands was in 1991 and was caused by toxigenic *C. diphtheriae*, one has to be aware of the possibility of diphtheria caused by toxigenic *C. ulcerans*.

SESSION 3

Microbiological surveillance of diphtheria
in the Newly Independent States (NIS) of
the former USSR

A 3.1: Laboratory diagnosis of diphtheria as part of diphtheria surveillance in Russia

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The procedures for laboratory confirmation of diphtheria in Russia have been developed and improved by several generations of researchers and clinical bacteriologists. The aim of laboratory analysis is the identification of the disease agent (toxigenic *Corynebacterium diphtheriae* only) within the shortest possible time.

In Russia, a shorter standard routine has been developed to bacteriologically diagnose diphtheria, comprising the minimum number of tests necessary, and sufficient to obtain a definitive and reliable result. The key test, which is the major component in microbiological diagnosis is to determine whether or not an isolated culture is capable of producing diphtheria toxin. Toxin production is identified on the second day of the bacteriological process when isolated colonies start to grow on blood tellurite agar, the selective culture medium used to isolate *C. diphtheriae*. Eliminating the stage of isolation on pure culture before conducting toxigenicity and cystinase tests will save one day. A Modified Elek test is performed with antitoxin (diphtheria antitoxic horse serum) purified with a specific absorption method to prevent non-specific precipitation lines. Biochemical tests, including cystinase, urease, glucose, saccharose, and starch decomposition tests, are performed. Nitrate tests are only carried out when there has been a positive urease reaction. Other tests are not feasible, since they add to the complexity, duration, and cost of analysis.

Molecular (PCR) and immunological (ELISA, ICS) tests may be used for provisional laboratory diagnosis of diphtheria for timely dissemination of appropriate intervention, including treatment. However, to obtain final confirmatory laboratory results, these methods must only be used in conjunction with the bacteriological method.

To ensure immunological control (determination of antitoxic diphtheria antibodies), an indirect haemagglutination assay (RPHA) is used. This method, used in conjunction with commercial test systems manufactured in Russia, proved to be the most appropriate method in a comparative study of the various methods used to determine antitoxic antibodies (RPHA, ELISA, vero cell, Jensen's *in vivo* method).

A 3.2: Levels of antitoxic antibodies and immunity to diphtheria amongst the population of the northwestern district of the Russian Federation in the post-epidemic period: 1996 to 2001

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The objective of this study was to determine the levels of anti-diphtheria antitoxin immunity in different population groups from areas with different incidence rates of diphtheria.

During the last five years, sera taken from 3800 adults and 4330 children from the territories of St Petersburg, Leningrad, Pskov region, and the Republic of Komi were studied. Starting in 1998, an annual increase of diphtheria incidence was observed in the first two regions, St Petersburg and Leningrad, with the highest levels seen in 2001 (4.5 and 3.5 per 100,000 population, respectively). In the two other regions, Pskov region and the Republic of Komi, a maximum of one case per year was observed. Despite active immunisation of the population, a high number of cases still occurred during the years that preceded this study due to the circulation of pathogens that had changed biotype and toxigenicity.

Three methods were used to determine levels of antitoxic antibodies (reaction of passive haemagglutination (RPHA), vero cell assay, and ELISA). The protective levels in four age groups were evaluated using international criteria.

The results showed that the levels of adult immunity varied yearly with the highest levels (>50%) observed between 1996 and 1997 in all age groups and 71% in those aged 20 to 29 years. This proportion changed between 1998 and 1999, with an increase in the percentage of individuals having only some degree of protection. Seronegative patients were found in all age groups (an average of 6% and 13% in individuals aged 50 years or over). There was an increase in the number of individuals with zero antibody levels in between 2000 and 2001 (an average of 18% and 31% in individuals aged 50 years or over). When a comparison was made between the populations in the four regions, it was observed that 76% of individuals were well protected in the Pskov region.

Adult aged 50 years or over with low levels of immunity are at higher risk of diphtheria than younger adults. In 2001, the increase in incidence may have been associated with the high proportion of adults

with insufficient immunity as well as the increased circulation of toxigenic *C. diphtheriae*.

When an evaluation of the efficacy of two booster doses of diphtheria vaccine in adults was carried out, protective levels were found in 46% of those vaccinated. They were also found after the follow-up period of four years, if the interval between immunisations was less than year. If the interval between the doses was two and more years, protective levels were only found in 21.6% after a further two years.

As the amount of time after the second dose increased, the proportion of non-susceptible individuals changed. After two years, high antibody concentrations were observed in 63% of individuals and the proportion with the lowest concentration was- 4%. However, after five years, the proportion of those individuals who were still well protected was 42% and no antibodies were detected in 11%.

It is necessary to monitor the immune status of the general population so that assessments can be made for future implementation of adequate vaccination programmes.

□ **A 3.3: Epidemic of diphtheria in Latvia**

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After a long period of 17 years, during which time no cases of diphtheria were reported in Latvia, new cases started to appear in 1986. At this time, diphtheria cases were also being reported in almost all former Soviet Union republics. Between September 1993 and 2001, diphtheria cases have been reported monthly in Latvia with 1288 cases of diphtheria in total during this time, including 96 deaths. Results of investigations conducted by local public health officials, immunisation histories, and hospital records, monthly records on morbidity of infectious diseases from local centres, were analysed. Surveys were organised to obtain data on risk factors. Diphtheria incidence peaked twice during this epidemic period: between 1994 and 1996 and between 1999 and the present time. During the first rise more than 71% diphtheria cases were reported among adults and especially high morbidity levels was registered among individuals aged between 30 and 59 years. The number of individuals in this group accounted for 75% of all adults. Seventy-nine per cent of cases were defined as severe clinical forms of the disease. In most cases, patients were unvaccinated. No fatal cases were reported among vaccinated patients. In order to prevent this diphtheria epidemic continuing, three immunisation campaigns were carried out in Latvia and coverage of vaccinated adults increased to 52%. During the second rise, the percentage of individuals with diphtheria aged between 30 and 59 years decreased by 19%. Thirty per cent of cases were registered among persons aged between 20 and 29 years, and 97% of them were vaccinated against diphtheria in childhood.

Currently, Latvia has the highest rate of diphtheria in Europe; the reasons for this resurgence are not completely understood. Unvaccinated individuals are at high risk of severe complications or death from infection with *Corynebacterium diphtheriae* while fully vaccinated or previously infected individuals are at low risk. Outbreaks of diphtheria can still occur among young adults living in close proximity, even if they are fully vaccinated. Booster doses with a higher antigen content received during adolescence may provide greater protection than the currently recommended toxoid.

A 3.4: Microbiological surveillance of *Corynebacterium diphtheriae* in Latvia

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The Laboratory of Microbiology in the National Environmental Health Centre is a national reference laboratory for diphtheria and therefore investigates all *Corynebacterium diphtheriae* strains isolated in Latvia. The laboratory conducts toxigenicity testing using PCR and the Elek test, biotyping, and antimicrobial susceptibility testing by ATB Expression produced by bioMérieux.

Toxigenicity of isolates are detected by a classical immunoprecipitation reaction in the Elek test and confirmed by the faster immunochromatographic test (ICS). Four hundred and fifty-nine and 134 isolates of *C. diphtheriae* were confirmed from patients, carriers, and close contacts in Latvia during 2000 and 2001, respectively. Eighty-seven per cent of these isolates were *C. diphtheriae* var *gravis*. Thirty-five per cent (24/69) of non-toxigenic isolates of *C. diphtheriae* had the *toxA* gene detected by PCR and 89% of these were var *mitis*.

Investigation of antimicrobial susceptibility showed that most *C. diphtheriae* strains were susceptible to the antimicrobials included in the ATB Strep 5 strip, although a few were resistant to penicillin, clindamycin, and cotrimoxazol. A small number (6/410) were resistant to two or three antimicrobials (*penicillin*, *erythromycin*, *rifampicin*, *tetracycline*). Ribotyping and phage typing of *C. diphtheriae* were carried out in collaboration with other diphtheria reference centres. The ribotypes St.Petersburg, Russia, and phage types VI and VI, with additional types 5 and 34 were the dominant strains in Latvia.

A 3.5: Current diphtheria situation in Smolensk region

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After the peak of the recent epidemic in the Russian Federation in 1994, there was a fall in the number of cases in all regions of Russia. However, during the last two years, some regions have reported another increase in the incidence of diphtheria. The objective of this study was to analyse the trends in diphtheria morbidity in the Smolensk regions during 2001 and compare them to those in 2000. A total of 17 diphtheria cases were reported in the Smolensk region in 2000 giving an incidence of 1.51 per 100,000 population (the incidence in the Russian Federation was 0.48 per 100,000 population). However, the numbers substantial increased by 76% in 2001 to 30 cases and the incidence was 2.66 per 100,000 inhabitants (the incidence in the Russian Federation was only 0.57 per 100,000 population). Seventy-three per cent of the cases were adults and, although there were no lethal cases reported in children, there were two adult deaths. Immunisation coverage rates in the Smolensk region of children under 3 years old and adult population were 98.5% and 95.1%, respectively. A dramatic increase in carriage rates of toxigenic *C. diphtheriae* strains was observed, from 0.6 per 100,000 population in 2000 to 5.2 per 100,000 population in 2001. Additional anti-epidemic measures are needed to increase immunization coverage rates in all age groups when there is an increase in the incidence of diphtheria.

A 3.6: Results of microbiological monitoring for diphtheria in the Ukraine: 1992 to 2001

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Improvements in the methods for laboratory diagnosis of diphtheria and an effective, functional system of microbiological monitoring has allowed the study of the populations of corynebacteria circulating in the Ukraine during the last decade to be carried out, as well as studies to confirm the substantial

heterogeneity. In spite of the predominance of toxigenic *Corynebacterium diphtheriae* var *gravis* during the epidemic years, an increase in the circulation of non-toxigenic *C. diphtheriae* var *mitis* was also observed. Furthermore, the circulation of *C. diphtheriae* var *belfanti*, including toxigenic variants, was observed for the first time. Using molecular and genetic studies, the circulation of epidemic strains in the Ukraine was confirmed. The role of these strains during periods of low incidence of disease can be speculated. However, there has also been widespread distribution of toxigenic and non-toxigenic *C. ulcerans* and *C. pseudotuberculosis* associated with upper respiratory tract infections and carriage.

A 3.7: Modern conditions of laboratory diagnosis of diphtheria in Tajikistan and retrospective analysis of data in dynamic of epidemic process

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A total of 11 diphtheria cases were reported in Tajikistan in 2000, including two deaths. Four of the cases were in non-vaccinated individuals and were imported. The ages of the cases varied, but five were observed amongst children aged between 7 and 14 years. A total of three diphtheria cases were reported in 2001 amongst non-vaccinated individuals in one region (aged 1, 6, and 22 years). All the strains were correctly transferred and confirmed at the Republican Sanitary and Epidemiological Station, showing that the procedures were carried out that allow for organised monitoring of diphtheria cases. The percentage of diphtheria cases confirmed by laboratory investigation in Tajikistan is 100%.

Action plans for the elimination of diphtheria for the period 2001 to 2010 were approved in Tajikistan and include the compulsory bacteriological investigation of all cases and contacts with transfer of isolated strains to the Republican Sanitary and Epidemiological Station, systematic monitoring of the performance of laboratories, and routine screening of throat swabs for diphtheria of all patients with pharyngitis (excluding catarrhal form) for the timely identification of patients and carriers of *Corynebacterium diphtheriae*.

Currently, the main problems facing diphtheria laboratories are the supply of media, the lack of screening of throat swabs in patients with pharyngitis in outpatient clinics and general hospitals, the absence of investigations in individuals from risk groups, and a lack of data on population immunity.

The Laboratory of the Republican Sanitary and Epidemiological Station performed retrospective analysis of the correlation of strains on age, region, and clinical aspects in both the pre-epidemic and post-epidemic

periods. It indicated some specific features in the development of diphtheria and formation of carriage. Analyses into the performance and conditions of laboratory services for the diagnosis of diphtheria may reveal factors that could potentially lead to increases in incidence.

A 3.8: Diphtheria in the Republic of Georgia

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The diphtheria epidemic in Georgia began in 1993 and the annual number of cases peaked at 429 in 1995. As a result of improved routine immunisation together with a massive immunisation campaign that started in 1995, a 33% decline in diphtheria cases was observed between 1995 and 1997. In 2000 and 2001, 28 and 22 cases were reported, respectively. Between 1996 and 2001, 6247 pharyngeal and nasal swabs were investigated. A total of 1236 strains were isolated, 1079 of which were toxigenic strains and 157 non-toxigenic.

Before 1998, *Corynebacterium diphtheriae* var *mitis* was the predominant strain isolated, but, since 1998, 67% of strains were *C. diphtheriae* var *gravis*. Only *C. diphtheriae* var *gravis* have been isolated since 1999.

Between 1993 and 1998, 66 strains of *C. diphtheriae* were characterised using the randomly amplified polymorphic DNA (RAPD) technique, pulsed-field gel electrophoresis (PFGE), and ribotyping. This study was described in a previously published article. Between 1999 and 2001, 30 toxigenic *C. diphtheriae* strains were isolated and identified by PCR. Molecular typing of toxigenic strains was performed by RAPD-PCR and PFGE. Two distinct genotypes have been identified and designated as RAPD₁ and RAPD₂ groups. Four clonal groups have been identified using PFGE.

Between 2000 and 2001, 1276 healthy children aged between 6 and 8 years and 12 and 14 years have been examined for *C. diphtheriae* carriage. Three strains of *C. diphtheriae* var *gravis* have been isolated (one toxigenic and two non-toxigenic strains). Susceptibility to penicillin, ampicillin, erythromycin, and ciprofloxacin was detected by the ATB 4 Strep (bioMérieux, France) for 25 strains isolated between 2000 and 2001. The majority of strains were found to be resistant to penicillin and erythromycin.

A 3.9: Current situation with diphtheria in St Petersburg, Russia

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The surveillance of diphtheria in St Petersburg is based on a combination of epidemiological, microbiological, and molecular typing data. Arbitrary primed PCR (AP PCR) with primer No.115 (as described by Bulat) is used as a screening method and ribotyping with *Bst* EI restriction enzyme and DIG-labelled OligoMix5 have been performed since 1994.

The dramatic increase in diphtheria incidence began in 1991 and reached its highest level of 52.1 per 100,000 population in 1994. During the epidemic, isolates from diphtheria patients and carriers were subjected to AP PCR which allowed 24 to 48 hour discrimination of a specific pattern designated g1. This particular AP PCR type has been identified in more than 95% of both toxigenic and non-toxigenic isolates of *Corynebacterium diphtheriae* var *gravis*. The g1 type included the ribotypes St Petersburg 1 and Murmansk 4a. These two ribotypes, indistinguishable by macrorestriction typing with *Sfi* I restriction enzyme and multilocus enzyme electrophoresis, were suggested to represent an 'epidemic' clone which caused diphtheria epidemic in Russia and its neighboring countries. Both these ribotypes accounted for the majority of all identified ribotypes in the St Petersburg area of northwestern Russia, with St Petersburg 1 predominating.

As a result of improved control of the epidemic in St Petersburg, the diphtheria incidence decreased to 1.4 per 100,000 by 1999. The distribution of clinical cases, the carriage of toxigenic, and the carriage of non-toxigenic *C. diphtheriae* has changed from 1:0.5:1.2 in 1994 to 1:0.9:4 in 1999. A tendency towards an increase of heterogeneity in the bacterial population was directly observed by the circulation of a larger number of different ribotypes in the general population of St Petersburg and represented an improvement in the epidemiological situation. However, an increase in diphtheria incidence (2.3 per 100,000) has been reported since 2000. It has been suggested that this is due to insufficient antitoxic immunity in the adult population aged 40 years and over. Since 2000, most diphtheria cases in adults have been caused by toxigenic *C. diphtheriae* var *mitis* ribotype Otchakov.

In 2001, the incidence of diphtheria reached 2.6 per 100,000 and was mainly in adult patients. Fifty-three per cent of cases were *C. diphtheriae* var *mitis* compared to 8% in 1994. Immunisation of the adult population is underway in the survey area, but it seems that ribotype Otchakov presents a new threat.

A 3.10: Microbiological control of diphtheria in the Republic of Moldova

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In the past, the incidence of diphtheria was always relatively low in the Republic of Moldova (0.2 per 100,000) as a result of mass immunisation and a high level of immunity in the population. An epidemic of diphtheria, however, commenced in 1992 (1.24 per 100,000) and the incidence increased to very high levels in 1994 and 1995 (22.83 per 100,000 in both years), after which it began to decrease (0.28 per 100,000 in 2001). This outbreak of diphtheria was due to a breakdown in the immunisation of the population between 1986 and 1990, which resulted in a large number of people with low immunity. Due to anti-epidemic measures, with the assistance of WHO, mass immunisation campaigns, and by monitoring the circulation of *Corynebacterium diphtheriae*, the level of immunity in the general population improved and the incidence of the disease fell. In 2000, diphtheria, once again, became a notifiable disease.

During the epidemic, cases occurred mainly in teenagers and adults, and the predominant biotype was *C. diphtheriae* var *gravis* (70%), followed by var *mitis* (28%) and var *intermedius* (2%). Non-toxigenic *C. ulcerans* was not observed in immunised individuals during this period. International collaboration is urgently required for the improvement of immunisation strategies and to enhance laboratory monitoring of the circulation of pathogenic corynebacteria and levels of immunity in the population of the Republic of Moldova.

A 3.11: Immunity to diphtheria from individuals from risk groups in the Kirov district of St Petersburg

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Beginning in 2000, a second epidemic wave of diphtheria was reported in St Petersburg, including the Kirov region. Seventy to 85% were observed amongst the adult population with toxigenic forms and lethal cases reported only in this group.

Screening and serological investigations among different social and professional risk groups including medical personnel, factory workers, and group with chronic tuberculosis were performed. A total of 472 sera from individuals aged between 20 to 69 years were studied. Antitoxic antibodies were determined using standard national (reaction of passive haemagglutination, RPHA) and international (Vero cell assay) methods. Immunity to diphtheria was evaluated according to WHO criteria for the protective levels of antibodies.

The study of the sera by RPHA showed that, in general, only 39% had protective levels of antibodies. In the group of medical personnel, 45% had protective levels, but, in the group of patients with tuberculosis, only 24%. Borderline immunity levels providing some protection (antibody titres 1:40-1:80) were found in 26% of studied individuals, with no differences in all studied groups.

Thirty-five per cent were susceptible to diphtheria (antibody titres from 0 to 1:20). Fifty-six per cent of susceptibles was found in patients with tuberculosis and, amongst this high percentage, 40% were seronegative. Amongst medical personnel, 28% were susceptible to diphtheria.

Amongst adults, the risk groups were patients aged between 50 to 59 years and 40 to 49 years. In these two groups, 52% of individuals had only ever been given one booster while the proportion who had been given two boosters was 25%.

Thus, immunological protection of adults in the district was not sufficient. Patients with tuberculosis are an important risk group. For effective control of the epidemic in this district, immunisation of adults with two boosters is required. In addition, the issue regarding the level of immunological protection of patients with tuberculosis should also be resolved.

SESSION 4

Clinical and microbiological aspects of
infection caused by potentially toxigenic and
other corynebacteria

A 4.1: Diphtheria antitoxin levels from three selected areas in Turkey

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Between February 2000 and March 2001, the Infectious Diseases Control Project in Turkey evaluated the diphtheria immunity status of populations from three selected areas, both urban and rural. The project is aiming to conduct the model survey for national sero-surveillance activity in Turkey.

The serum samples were obtained from 1018 randomly selected healthy subjects aged between 0 and 82 years, divided according to sex and age groups, and the diphtheria antitoxin levels of the sera were measured by *in vitro* toxin neutralisation test on Vero cells.

Only 13% of subjects had <0.01 IU/ml diphtheria antitoxin, indicating susceptibility to diphtheria, 19% had levels ≥0.01 and 0.09 IU/ml, indicating basic protection, and 68% had levels ≥0.1 IU/ml indicating full protection according to internationally accepted criteria. The immunity level was high in subjects less than 20 years of age, decreased in those aged between 20 and 40 years, and then increased in those aged over 40 years. Therefore, booster immunisation with diphtheria toxoid may be advisable for the adult population.

A 4.2: Levels of anti-pertussis antibodies in comparison to diphtheria antitoxin levels in the Greek population

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Antibody titres to pertussis toxin (PT) and filamentous haemagglutinin (FHA), which arise from vaccination as well as natural infection, were compared with diphtheria antitoxin (DAT) levels in sera from 429 healthy children and adults aged between 0 and 80 years in Northern Greece. The concentration of IgG antibodies to PT and FHA were measured by an ELISA test using a mixture of purified PT and purified FHA antigens of *Bordetella pertussis* Toham Phase I strain. Antibody levels were considered elevated when they were higher or equal to 9 ELISA units. The serum levels of diphtheria antitoxin were determined by IgG-specific ELISA (Virotech System-Diagnostika GmbH). An antitoxin concentration of ≥ 0.1 IU/ml was considered to be at a minimum protective level. A regression analysis model on IgG in the logarithmic scale with age as the explanatory variable was used for levels of anti-pertussis antibodies. For anti-pertussis antibodies $\log(\text{IgG})$ was significantly increasing with age ($p < 0.001$), which was in contrast to the findings with diphtheria antitoxin levels. When age was treated as a variable with nine categories, analysis of variance showed significant differences of $\log(\text{IgG})$ between age categories. The only significant difference in $\log(\text{IgG})$ levels detected according to the Scheffe Post Hoc Tests was between 5 and 10 years and 41 and 50 years ($p = 0.007$). Immunity rates against diphtheria were found to be protective in high proportions of northern Greek children up to 20 years while above this age the DAT levels declined sharply. These findings indicate that immunity of children against pertussis due to basic immunization, in contrast to diphtheria immunity, is not adequate even in the first two years of life. It is possible that the whole cell DTP vaccines in use during the period of blood sampling were of low efficacy. Also, antibody titres were relatively low in those aged between 5 and 10 years. This finding supports information about the vaccination status showing that a fifth booster pertussis vaccine dose is not given after the second year of life in most individuals.

A 4.3: Seroprevalence of diphtheria immunity among injured adults in Austria

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During a one-year period, a seroepidemiological study was conducted at an outpatient clinic of a trauma department. Immunity to diphtheria was determined in serum samples from 558 injured patients (205 women and 353 men, age between 18 and 70 years). Diphtheria-antitoxin concentrations were measured with an enzyme immunoassay and a tissue culture toxin-neutralisation assay. Sero-immunity was classified as susceptible (<0.01 IU/ml), basic protection (0.01 – <0.1 IU/ml), and full protection (≥ 0.1 IU/ml) against the toxic manifestations of the disease. Twenty-seven per cent of the subjects were susceptible to diphtheria, 27% had basic protection, and 46% were fully protected. The median antitoxin concentration was 0.08 IU/ml (0.0 - 0.29; quartiles Q25 - Q75). A non-linear trend toward decreasing immunity with increasing age was observed ($p < 0.001$) and it was observed that females were less protected than males ($p = 0.006$). The country of previous immunisation (Austria, Western European countries, Eastern European countries, and non-European countries) had no influence on sero-immunity ($p = 0.49$). Multiple linear regression analysis revealed that age ($p < 0.001$) and gender ($p = 0.004$) had a significant independent influence on diphtheria immunity level, whereas the country of previous immunisation was not significant ($p = 0.72$).

A 4.4: Penicillin tolerance of non-toxicogenic *Corynebacterium diphtheriae* strains isolated from cases of pharyngitis

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Current guidelines state that isolates of non-toxicogenic *Corynebacterium diphtheriae* should be regarded as potential pathogens and patients should be treated with penicillin or erythromycin if symptomatic. A total of 24 strains of non-toxicogenic *C. diphtheriae* var *gravis*, isolated from the throats of patients who presented with pharyngitis and tonsillitis during an enhanced surveillance study, were assayed for sensitivity to penicillin and erythromycin. Seventy-one percent (17/24) of strains were shown to be tolerant to penicillin. There were no differences in the minimal growth inhibitory concentration of penicillin between susceptible and tolerant strains. However, minimal bactericidal concentration values were ≥ 2 -4 mg/L in 96% of the strains (23/24), which corresponded to the penicillin serum level. In contrast, all strains were susceptible to erythromycin. Therefore, these aspects should be considered when selecting therapy for the treatment of 'non-toxicogenic' *C. diphtheriae* sore throat.

A 4.5: Antibiotic-resistant *Corynebacterium diphtheriae* strains circulating in Russia

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The wide use of antibacterial drugs to treat diphtheria patients and carriers frequently impairs local oropharyngeal and nasal immunity and microbial flora. Persistent presence of *Corynebacterium diphtheriae* in a diphtheria carrier, where repeated drug therapy does not result in agent elimination, causes the strain to become drug-resistant. This study describes the antibiotic sensitivities in *C. diphtheriae* strains isolated in Russia.

One hundred and forty-eight strains of *C. diphtheriae* were studied, including 118 toxigenic and 30 non-toxigenic isolates from diphtheria patients and carriers in Moscow, Murmansk, Omsk, Kaliningrad, Vladimir and Krasnodar between 1998 and 2000. Sixteen strains (11%) were found to be resistant to macrolide and lincosamide antibiotics such as erythromycin, azithromycin, lincomycin, and clindamycin. Minimal inhibitory concentrations exceeded 256 mg/l. Resistant strains were characterised as toxigenic, *C. diphtheriae* var *gravis*, Sankt-Peterburg/Rossija ribotype (epidemic clonal group of strains). No antibiotic-resistant strains were found among those that circulated in Russia from the early 1980s to 1998. All the antibiotic-resistant strains were collected in a period of low incidence that followed the period of high incidence when high numbers of carriers were subjected to repeated antibiotic treatment, primarily with erythromycin.

An impairment of the upper respiratory tract immune mechanisms and microflora, ineffectiveness of antibiotic treatment of diphtheria carriers, and the emergence of antibiotic-resistant strains resulting from the wide use of antibiotics, all call for explicit guidelines to regulate drug prescription in an effort to control diphtheria infection. At the same time, drug-resistant strains need to be further monitored, and mechanisms of their drug-resistance need to be studied in more depth.

A 4.6: Susceptibility to antibiotics of some *Corynebacterium diphtheriae* strains isolated in the Newly Independent States (NIS)

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Six hundred and ninety-two strains of *Corynebacterium diphtheriae* from the following Newly Independent States (NIS) countries: Belarus, Latvia, Moldova, and Russia were studied as part of the microbiological surveillance of diphtheria. The strains were laboratory confirmed and typed by phage typing. One hundred and forty-two of the strains from patients, contacts, and healthy carriers were investigated for susceptibility to antibiotics using the agar dilution method. The strains of *C. diphtheriae* were generally susceptible to: amoxicillin, penicillin, cefuroime, gentamicin, tetracycline, ciprofloxacin, ofloxacin, chloramphenicol, and erythromycin. Other authors have also made this observation, on strains isolated from the same epidemic

area. In varying degrees, there was evidence of resistance to trimethoprim (CMI>8mg/l) and rifampicin (CMI>16 mg/l) in some of the strains. The majority of the strains were resistant to rifampicin and represent a change of the phages in the susceptibility spectra. These rifampicin-resistant strains were isolated both in the Republic of Moldova and in the bordering counties of Romania, and some were non-toxigenic *C. diphtheriae* strains that had been isolated from angina cases.

A 4.7: The characteristics of some biological properties of *Corynebacterium diphtheriae*

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The biological properties of 170 isolates of *Corynebacterium diphtheriae* were examined. Standard control cultures of corynebacterium were also examined and included: *C. diphtheriae* var *gravis* NCTC 10648, *C. xerosis* 1278, *C. diphtheriae* var *mitis* 39/9; *C. ulcerans* 09496; *C. pseudodiphthericum* 92 (Russia, SISC).

Most isolates were typical of their species and biotype however some atypical reactions were documented and included fermentation reactions for arabinose, xylose, galactose, maltose, and glucose, the methyl red reaction, as well as casein and tyrosine hydrolysis. Four per cent of the isolates were negative for nitrate reduction and therefore, belonged to the biotype *C. diphtheriae* var *belfanti*.

While analysing the differences in the biological properties of toxigenic and non-toxigenic *C. diphtheriae*, it was found out that non-toxigenic isolates varied to a greater extent from typical biological reactions. Statistically significant differences in the fermentation reactions of non-toxigenic strains were observed. This extensive study of the physiological and biological attributes of 170 cultures of *C. diphtheriae* therefore revealed that many properties such as cystinase, pyrazinamidase, urease, and fermentation reactions are more stable for toxigenic strains.

A 4.8: Use of cell wall antigens of non-toxigenic *Corynebacterium diphtheriae* in an ELISA to determine diphtheria antibodies in human sera

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Antitoxic antibodies play an important role in protection against the clinical disease of diphtheria. However, this is not the only immune response that is produced during infection, as it ignores the role that bacterial antigens play in antibody production.

Carriage of toxigenic, as well as non-toxigenic, *C. diphtheriae* can still occur when there are high levels of antitoxin in the blood of an individual. The sera of both healthy individuals and asymptomatic vaccinated carriers of toxigenic *C. diphtheriae* were examined using the ELISA test to measure antibody levels.

This population-based serological analysis was used to study the role of antibacterial IgG, IgM, and IgA antibodies types. The relationship between antibacterial immunity and carriage of diphtheria has been established, as well as the level of antibodies before and after vaccination of people by diphtheria antitoxin.

A 4.9: Invasion of cultured human epithelial cells by *Corynebacterium diphtheriae*

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Disease due to *Corynebacterium diphtheriae* can take different clinical forms. Toxigenic strains are responsible for the clinical disease of diphtheria, but immunised patients may have bacteraemia and endocarditis in the absence of the characteristic toxin-mediated lesions. Non-toxigenic strains have been increasingly recognised as a cause of the infectious process that may range from cutaneous lesions and pharyngitis to severe invasive diseases (endocarditis, osteomyelitis, and arthritis). The upper respiratory pharyngeal mucosa is the primary site of colonisation by this microorganism. Besides toxin production,

little is known about other *C. diphtheriae* virulence factors. Previous studies have dealt with the adhesion of *C. diphtheriae* to human buccal epithelial cells and erythrocytes. Bacterial adhesion properties are independent of toxin production. In this study we evaluated the adhesive and invasive capacity of non-toxigenic strains by infecting human cells with strains that have been isolated from severe cases of pharyngitis. All strains tested adhered to, and invaded, human pharyngeal epithelial cells (Detroit D562, ATCC CCL-138). The adherence and invasion efficiency were measured: the number of adherent bacteria measured after one hour incubation at 37°C corresponded to 7.15 cfu/100 cells and intracellular bacteria at three hours and 24 hours were 0.18 and 0.1 cfu/100 cells, respectively. The internalised bacteria survived for at least 24 hours although replication seemed to occur only occasionally. We found that cytochalasin D almost completely inhibited internalisation of all bacterial strains, whereas colchicine had no effect, indicating that host microfilaments play a major role in bacterial internalisation. The use of the lysosomotropic agent ammonium chloride demonstrated that a pH increase in the intracellular vesicles did not affect bacterial entry. Transmission electron microscopy showed corynebacteria penetrating inside cells through non-clathrin coated membrane invaginations and, inside the cytoplasm, vacuole membranes tightly surrounded bacteria. Bacterial cells did not seem to undergo enzymatic degradation up to 48 hours and cells were never observed free in the cytoplasm. These results indicate that *C. diphtheriae*, as well as *Streptococcus pyogenes*, can be internalised by non-professional phagocytic cells. The relevance of this phenomenon deserves further investigation.

A 4.10: Patterns of adherence to HEp-2 cells and the ability to induce actin polymerization by toxigenic *Corynebacterium diphtheriae* strains

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The adherence of a microbe to tissue or cells precedes all infectious diseases. Little is known about the colonisation factors of *Corynebacterium diphtheriae*, the receptors they recognise, or the relative contributions of filamentous and/or cell surface adhesins. This investigation was undertaken to examine the interactions leading up to and involving the adherence process of *C. diphtheriae*, now commonly considered the primary step bacteria make in colonising, invading or causing disease. Thirteen strains were tested and displayed varying degrees of attachment to HEp-2 cell monolayers with two distinct adherence patterns, termed localised (LA) and diffuse (DA). The DA phenotype predominated over the LA phenotype. The LA pattern was mainly observed among the glass adherent/sucrose fermenting strains. The fluorescein isothiocyanate (FITC)-labelled phalloidin assay (FAS test) was used as indicative of the ability to induce actin polymerisation by *C. diphtheriae*. Transmission electron microscopy demonstrated

cellular lesion similar to the histopathology seen with enteropathogenic *Escherichia coli* (EPEC) strains in which the bacteria adhere in an intimate fashion causing actin polymerisation and sometimes sit upon a pedestal-like structure. Fluorescence microscopy revealed that non-fimbrial protein (67-72p) interacted directly with HEp-2 cell membranes. Bacterial adherence to HEp-2 cells was inhibited in varied degrees by carbohydrate moieties. The *N*-acetylneuraminic acid residues (10% final concentration) gave total inhibition of adherence of two strains tested. Initial indications point to 67-72p and carbohydrate components as putative adhesins of *C. diphtheriae* to human epithelial cells. The regulation of the expression of these extracellular structures and their individual roles in the adhesion process remains to be determined.

A 4.11: Non-toxigenic corynebacteria associated with human infection: emerging pathogens

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For many years, the pathogenic potential of coryneform bacteria other than *Corynebacterium diphtheriae* has been underestimated. Only in the last few decades have these bacteria been associated with human disease. As the survival of severely compromised patients increases with advances in health care, clinical microbiologists are more frequently encountered opportunistic coryneform commonly called diphtheroids. The association of some corynebacteria with particular diseases are promoting identification of these bacteria in pathogenic situations. The genus *Corynebacterium* sp is the largest genus in the coryneform group, which includes aerobically growing aspoxygenous, non-partially-acid-fast, irregular, Gram positive rods.

The Special Bacteriology Division of National Institute of Infectious Diseases of Argentina received eighty-nine clinical isolates of coryneform organisms in one year. The organisms were identified by biochemical analysis based on a comprehensive study in which phenotypic methods were used. The coryneforms investigated were identified as: *C. xerosis* 23% (21/92), *C. urealyticum* 22% (19/92), *C. afermentans* 15% (14/92), *C. jeikeium* 9% (8/92), *C. pseudodiphtheriticum* 7% (6/92), *C. amycolatum* 5% (5/92), *C. ulcerans* 3% (3/92), *C. coyleae* 3% (3/92), CDC group G 3% (3/92), CDC group F-1 2% (2/92), *C. pseudotuberculosis*, *C. accolens*, "*C. aquaticum*" and *C. propinquum* 1% (1/92) one each, *Aureobacterium* sp 1% (1/92), *Arcanobacterium haemolyticum* 2% (2/92), and *Oerskovia xanthineolytica* 1% (1/92).

The bacteria identified were isolated from blood culture (37%), urine (21%), catheter (7%), spinal fluid (6%), respiratory tract (4%), eye conjunctiva (3%), abscess (7%), and 21% other sites such as wound, osteomyelitis, granuloma, etc.

it should be mentioned, finally, that the inability of a microbiology laboratory to accurately isolate and identify coryneform bacteria could reduce the recognition of its potential significance.

SESSION 5

Molecular and genetic characteristics
of corynebacteria
Symposium sponsored by the Wellcome Trust

A 5.1: The *Corynebacterium diphtheriae* strain NCTC 13129 genome project

A M Cerdeño, J Parkhill, and The Pathogen Sequencing Unit

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The *Corynebacterium diphtheriae* strain NCTC 13129 genome is being sequenced at The Pathogen Sequencing Unit in The Sanger Institute. NCTC 13129 is a recent clinical isolate that is representative of an epidemic clone now circulating within the Eastern European region. The chromosome was sequenced using a whole-genome shotgun technique, with the final sequence being assembled from 66,099 sequencing reads. The complete genome sequence is now finished, and is undergoing analysis and annotation. The genome of *C. diphtheriae* is 2,488,635 bp long and is predicted to contain 2,382 coding sequences. The annotation has revealed a number of interesting features of the genome and will hopefully be able to give an insight into the life cycle and pathogenicity of this bacterium. The latest results of the analysis of *C. diphtheriae* will be presented. Sequence data from this project is available from http://www.sanger.ac.uk/Projects/C_diphtheriae/.

A 5.2: Whole genome visualisation, analysis, and comparison using Artemis and ACT

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Analysis and annotation of bacterial genomes at The Sanger Institute is carried out using the software tool Artemis. Artemis is designed to allow the results of any analysis or set of analyses to be viewed in the context of the sequence, and its six-frame translation. Sequence and annotation data can be loaded directly from EMBL or GenBank format files. The program is written in Java, and is thus able to run on Unix, linux, PC and Macintosh systems, and is freely available under the GNU software licence from www.sanger.ac.uk/Software/Artemis. An extension of Artemis, the Artemis Comparison Tool (ACT) allows full pair-wise comparisons between any number of genomes, at the DNA or protein level, to be viewed in a fully interactive manner. As with Artemis, the program can

be run on virtually any available system to view the results of pre-run comparisons. The use of Artemis and ACT will be demonstrated, with particular reference to the analysis of *Corynebacterium diphtheriae* with a comparison to related genomes.

A 5.3: Toxigenic and non-toxigenic *Corynebacterium diphtheriae* in Turkey: characterisation of strains by using biological and molecular typing methods

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Biotyping, toxigenicity testing with the ELEK test and PCR, randomly amplified polymorphic DNA (RAPD) analysis (Ready-to-Go RAPD kit, Primer 4, Amersham Pharmacia, NJ) and pulsed-field gel electrophoresis (PFGE) have all been employed in the characterisation of *Corynebacterium diphtheriae* strains circulating in the south-east part of Turkey. Four cases and 434 contacts have been investigated in our laboratory since August 1999. Two of the patients died with respiratory obstruction. In this study, 12 isolates from cases and their contacts were investigated. Toxigenic *C. diphtheriae* var *mitis* was isolated from two cases and three contacts. One of the cases was found to be colonised by non-toxigenic *C. diphtheriae* var *gravis* at the same time. In addition, non-toxigenic *C. diphtheriae* var *gravis* was isolated in three of the contacts and non-toxigenic *C. diphtheriae* var *mitis* in another two. Five RAPD types and three PFGE patterns (by using *Sfi*I restriction endonuclease) were identified among the strains. Identified biotypes and toxigenicity characteristics were completely compatible with the PFGE patterns. The PFGE patterns among the toxigenic strains did not vary, even when the strains were isolated 6 to 8 months apart and in different regions of the country. No epidemiological relationship could be ascertained from the RAPD types of the isolates.

A 5.4: Genotype characteristics of *Corynebacterium diphtheriae* strains in a period of low diphtheria incidence in Russia: 1997 to 2001

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Between the 1940s and 1990s, 17 different ribotypes of toxigenic *Corynebacterium diphtheriae* strains have been isolated in Russia. Despite this genetic heterogeneity of the diphtheria agent, during each epidemic cycle only one particular biovariant and ribotype prevailed.

In the 1940s and 1960s, for example, the 'Lyon' ribotype of *C. diphtheriae* var *gravis* prevailed, in the 1980s it was the 'Otchakov' ribotype of *C. diphtheriae* var *mitis*, and the most recent epidemic of the 1990s was characterised by the prevalence of the closely related 'Sankt-Peterburg' and 'Rossija' ribotypes of *C. diphtheriae* var *gravis*. Circulation of *C. diphtheriae* strains of 'Sankt-Peterburg' and 'Rossija' ribotypes increased between 1986 and 1989, signaling the 'unfriendly' situation of an upcoming epidemic rise.

After 1997, during a period of lower incidence, the structure of the *C. diphtheriae* population, homogeneous in the years of the epidemic, started showing a new trend. Whereas in 1996, 96% of all strains were of the epidemic group ('Sankt-Peterburg' and 'Rossija' ribotypes), by 1997 the proportion decreased to 87%, with 88% in 1998 and 1990, and, finally to 77% in 2001. As 'Sankt-Peterburg' and 'Rossija' ribotypes become less common, other ribotypes such as 'Otchakov', 'Buzau', 'Lyon', 'Cluj', G 4v, and a further 15 types, are becoming more prominent. However, it would be premature to describe this as a 'friendly' situation, since circulation of *C. diphtheriae* var *gravis* is proceeding at a very high rate (70% *gravis* and 30% *mitis* in 2001), continuing to preserve epidemic strains.

Monitoring phenotype and genotype properties of *C. diphtheriae* strains in periods of relatively low disease incidence may hopefully allow identification of new epidemically significant strains and prediction of future epidemic processes.

A 5.5: Etiological value of non-toxigenic *tox*-bearing (NTTB) *Corynebacterium diphtheriae* strains

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The role of diphtheria toxin in the pathogenesis of diphtheria is widely known. Recently, a number of non-toxigenic *C. diphtheriae* strains have been reported to contain the *tox*-gene but have produced no diphtheria toxin. Some researchers have suggested that these strains may restore their toxigenic properties after a passage on nutritional media, and therefore should be considered of etiological importance. They therefore suggest including these strains into diphtheria surveillance.

We have undertaken a comprehensive study of non-toxigenic *tox*-bearing (NTTB)-strains and found that they all belong to *C. diphtheriae* var *mitis*, are homogeneous in their pheno- and genotype properties, and differ from toxigenic *C. diphtheriae* strains in their genetic structure. NTTB-strains do not produce either diphtheria toxin or any of its fragments, which is explained by the mutation – a single base deletion at nucleotides 52-55 resulting in a frameshift and subsequent loss of the *tox* open reading frame and the truncation of the *tox* protein. Therefore, NTTB-strains are truly non-toxigenic strains unable to cause diphtheria. The fact that some researchers have discovered toxigenic properties in these strains may be explained by their mistaking low toxin production or delayed toxin production strains for NTTB-strains (with the former being indeed capable of easily restoring their toxigenic properties after a passage on nutritional media). On the contrary, NTTB-strains cannot be induced to restore their toxigenic properties under any *in vitro* or *in vivo* cultivation settings. We have developed a method to differentiate between NTTB-strains and toxigenic strains of *C. diphtheriae* or *C. ulcerans*. The method is based on endonuclease restriction of *tox*-gene PCR-amplicons.

One must bear in mind the possible role of NTTB-strains as a *tox*-gene reservoir. In light of this, it is important to continue monitoring their circulation and their properties.

A 5.6: Interstrain heterogenicity of the non-toxigenic *tox*-bearing (NTTB) *Corynebacterium diphtheriae* strains

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The heterogenicity of pathogenic *Corynebacterium diphtheriae* strains is an established fact, however there are variants among *C. diphtheriae* that contain the *tox*-gene but produce no toxin. Two hundred and seventy colonies of one non-toxigenic *tox*-bearing (NTTB) *C. diphtheriae* var *mitis* strain were studied in order to research interstrain heterogenicity. NTTB-strains may restore their toxigenic properties after passage on nutritional media. The impact on toxin production of these strain made by other toxigenic strains via dialysis cellophane, as well as pure toxin of different concentrations, was observed. Toxigenicity testing of the discrete colonies, after passage through dialysis cellophane, was carried out by the immunochromatographic strip test (ICS) and Elek test.

Thirty-two per cent (87/270) of the colonies produced toxin after two to three passages, determined by ICS and Elek test. The ICS test was able to test for toxin production one passage before the Elek test, due to its increased sensitivity compared to the immunoprecipitation method. Sixty-eight per cent of the colonies remained non-toxigenic after five to six passages. Restoration of toxin production in these colonies was different under the impact of pure toxin of different concentrations. Haemolytic activity became stronger and morphological characteristics after passage.

In a natural ecological niche, conditions only arise for toxin production of certain colonies. This study of the NTTB-strains showed that there is interstrain heterogenicity and, after passage through dialysis cellophane, there is restoration of toxin production in one variant while, in the other, there is no restoration, even after six passages.

A 5.7: Use of transposon mutagenesis to analyze regulation and function of the diphtheria toxin repressor in *Corynebacterium diphtheriae*

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Under high-iron conditions, the diphtheria toxin repressor (DtxR) represses both the *tox* gene that encodes diphtheria toxin (DT) and other genes required for siderophore biosynthesis, siderophore-dependent iron uptake, and assimilation of iron from haem. Few tools are currently available for genetic analysis of *Corynebacterium diphtheriae*. We developed a method for transposon mutagenesis in *C. diphtheriae* and used it to isolate two mutants with decreased production of DtxR. Mutant 1 contains an insertion within the *dtxR* gene and produces no DtxR, indicating that DtxR is not essential for viability of *C. diphtheriae*. As a consequence of lacking DtxR, mutant 1 constitutively produces DT and siderophore; in addition, it exhibits enhanced susceptibility to oxidative stress. Mutant 2, which contains a transposon insertion in the intergenic region located upstream from *dtxR* and downstream from an open reading frame that is predicted to encode a sigma factor, produces wild type DtxR in smaller amounts than the parental strain. Mutant 2 produces DT but not siderophore under high-iron conditions, supporting previous suggestions that siderophore biosynthesis is more stringently repressed by DtxR than is DT production. We also showed that wild type *C. diphtheriae* produces at least two transcripts that encode *dtxR*, one that includes the sigma gene plus *dtxR*, and one that encodes only *dtxR*. Our identification of two different transcripts that encode *dtxR* provides the first evidence that multiple mechanisms may control the production of DtxR and virulence gene expression in *C. diphtheriae*.

A 5.8: Adaptation of the international *Corynebacterium diphtheriae* ribotypes database to RiboPrinter

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The resurgence of diphtheria in Eastern Europe induced the World Health Organization (WHO) Regional

Office for Europe to create a European Laboratory Working Group on Diphtheria (ELWGD) and to initiate the designation of national reference laboratories on diphtheria in as many countries as possible in the WHO European Region. One of the objectives of the surveillance of diphtheria in Europe was to establish a ribotyping database for comparing strains in the different countries. In an earlier study, a total of 595 strains of *Corynebacterium diphtheriae* were submitted to a manual ribotyping technique and the different ribotypes obtained were analysed by computer with the software programme Taxotron® (Taxolab, Institut Pasteur). To date, 86 distinct ribotypes with the endonuclease *Bst*Ell were chosen for the ribotyping database, and each ribotype pattern was represented by a reference strain possessing an unique geographical name, producing a stable and reproducible ribotype pattern. These reference strains had also common ribotype patterns generated by both endonucleases *Bst*Ell and *Pvu*II.

These strains were now submitted to automated ribotyping using the RiboPrinter® Microbial Characterization System (Qualicon, Wilmington, DE, USA) and endonucleases *Bst*Ell and *Pvu*II. Gel images were exported in TIFF format and computer analysed with programs of the Taxotron package. The cubic Spline algorithm was used for fragment size calculation.

A schematic representation of all reference ribotype patterns can facilitate the identification of ribotypes in different laboratories, in spite of the fact that patterns obtained with the RiboPrinter did not show the same band resolution as patterns obtained manually. Our study shows that automated ribotyping can type *C. diphtheriae* isolates routinely and quickly, which is very useful in a national reference centre and can facilitate the recognition of new ribotype patterns.

A 5.9: Molecular characterisation of clinical isolates of *Corynebacterium diphtheriae* isolated from northern states of India including Delhi

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Between 2000 and 2002, 30 isolates of *Corynebacterium diphtheriae* were recovered from clinical specimens obtained from suspected cases of diphtheria that had been admitted to the Infectious Diseases Hospital, Delhi, India. These isolates were characterised by conventional biochemical tests as well as by using a commercial kit (API Coryne system, bioMerieux, France). The isolates were toxigenicity tested by the Elek immunoprecipitation test. In addition, the isolates were typed by SDS-PAGE whole-cell protein profiling analysis and ribotyping using a digoxigenin labelled cDNA probe synthesised by employing 16S+23S r-

RNA of *E. coli*. The protein and riboprofiles were analysed by computerised analysis using 'Diversity' software (pdi, USA) incorporated in a gel documentation system. Twenty-eight of the 30 isolates were identified as *var intermedius*, while the remaining two were *var mitis*. Twenty-six of the isolates were toxigenic strains, while all of the remaining four isolates were non-toxigenic *C. diphtheriae var intermedius*. These findings reveal a shift from *var mitis* to *var intermedius* as the predominant biotype in India. Eighty-seven per cent of the isolates in the present study were found to be toxigenic. This was a significant change from the previous years, when in 1981 almost 100% of the strains encountered were non-toxigenic. The shift in the predominant biotype may be related to a resurgence of diphtheria in India. The isolates could be divided into 14 protein types by SDS-PAGE whole-cell protein profile analysis. Eight of the isolates had the same protein type. By ribotyping, the isolates could be ascribed to 17 types. A cluster of seven strains of one ribotype was encountered. This clone was the predominant clone encountered in Delhi and is not found in any of the neighbouring states of Harayana, Uttar Pradesh, and Rajasthan. The discriminatory indices of protein typing and ribotyping were calculated to be 0.89 and 0.92, respectively. Ribotyping was, therefore, found to be an efficient, highly reproducible, and discriminatory technique for molecular typing of clinical isolates of *C. diphtheriae*.

A 5.10: Molecular characterisation of non-toxigenic *tox*-gene bearing corynebacteria strains

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The emergence of non-toxigenic *tox*-gene bearing corynebacteria isolates has become an increasing problem in the field of epidemiology.

Strains of *Corynebacterium diphtheriae* and *C. ulcerans* isolated in Romania from 1962 until 2000, in Russia and Republic of Moldova during the diphtheria epidemics, and in Republic of Moldova in 2000, were identified in a previous study as ELEK negative and PCR positive for *tox*-gene. These strains were analysed using different molecular approaches including ribotyping, toxigenotyping, and RFLP of a 910 bp PCR product, in order to find some characteristics useful as discrimination markers.

Ribotyping was performed after *Bst*II restriction and hybridisation with 5 oligo mix-DIG labelled probe. Toxigenotyping method was based on the pattern of hybridisation of fragments of total DNA digested with enzymes with a various number of restriction sites within *dtox* gene. A 910 bp-DIG labelled amplification product, homologous to the *dtox*-gene sequence, was used as a probe. *Pvu*II, *Cl*I, *Hin*II and *Mbo*II were used for RFLP of the 910 bp PCR product and the restriction patterns were analysed and compared in order to define genetic changes in *dtox* gene sequence.

A 5.11: Molecular epidemiology of *Corynebacterium diphtheriae* and *C. ulcerans* strains isolated in Italy

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Strains of *Corynebacterium diphtheriae* and *C. ulcerans* isolated in Italy during the 1990s were characterised by biotyping, toxigenicity testing, and ribotyping. Five cases of diphtheria were reported during the last ten years; four were due to infection with *C. diphtheriae* and one to *C. ulcerans*. During the same period, a total of 11 cases of infection with non-toxigenic *C. diphtheriae* were reported to the Italian Public Health Institute.

Ribotyping of a toxigenic strain of *C. diphtheriae* isolated from a fatal case of diphtheria that occurred in an immigrant showed a unique band pattern, not previously seen among isolates of *C. diphtheriae* *var gravis*. Therefore, it was assumed that the strain was imported from South America.

Diphtheria caused by *C. ulcerans* is a rare disease but, in recent years, several cases have been registered in the United Kingdom (UK). Interestingly, the only toxigenic strain isolated in Italy produced a ribotype indistinguishable to that produced by UK isolates of *C. ulcerans*. The significance of this is unclear, as there were no definitive epidemiological links with the UK. The dissemination of *C. ulcerans* strains within the European Region or indeed globally is currently unknown.

Molecular typing of non-toxigenic strains that were mainly isolated from patients that presented with fever, severe pharyngitis, and tonsillitis, produced several distinct ribotype patterns. The *var gravis* isolates produced two distinct ribotypes provisionally designated as 'D11' and 'D75'. Isolates of *var mitis* produced two distinct patterns, provisionally designated as 'D76' and 'D77'. The ribotype 'D11' had been previously documented amongst non-toxigenic *var gravis* strains isolated in the UK, Russia, Germany, Romania and Sweden. Ribotype 'D75' was also seen amongst non-toxigenic *var mitis* UK isolates whilst the other ribotypes had not been documented anywhere else.

A 5.12: Characterisation of toxigenic *Corynebacterium ulcerans* strains isolated in the UK from humans and domestic cats

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Corynebacterium ulcerans is a veterinary pathogen and causes mastitis in cattle and other domestic and wild animals. Toxigenic strains of *C. ulcerans* have been associated with classical diphtheria as well as milder symptoms. At least one death within the UK has been attributed to such an infection. Usually human infections are acquired through contact with animals or by ingestion of unpasteurised dairy products. Person-to-person transmission of *C. ulcerans* could be possible and it has been recommended that the public health response to human infection with *C. ulcerans* should be the same as for *C. diphtheriae*.

In the UK, the frequency and severity of *C. ulcerans* infections appear to be increasing. Between 1986 and 2001 a total of 51 *C. ulcerans* isolates were submitted to the PHLS Streptococcus and Diphtheria Reference (SDRU) Unit for identification. Forty-three (84%) of the isolates were toxigenic. However, the source of human *C. ulcerans* infection in the UK is not often determined. Recently, toxigenic *C. ulcerans* has been isolated from three cats with bilateral nasal discharge. The organism had not been identified previously in cats. Therefore, cats with nasal discharge may represent a source of human infection. Ribotyping results have shown that the profiles produced by isolates from the cats are, thus far, indistinguishable from human isolates. Tests, including 16s rRNA sequencing, have been undertaken to confirm both the species identification and other characteristics.

A 5.13: Travellers as human reservoirs of *Corynebacterium diphtheriae* – a story between England and Israel

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A toxigenic strain of *Corynebacterium diphtheriae* var *mitis* was isolated by the Public Health Laboratory Service (PHLS), London on 14 January 2002 from a throat swab of a boy aged 11 years old from a religious community in Salford, northwest England. The child had recently returned from a one-week holiday in Jerusalem, Israel with four members of his family. The Central Government Laboratories in Israel were notified on 16 January 2002, and throat swabs were taken from seven members of the Israeli family: three adults and four children. A toxigenic strain of *C. diphtheriae* var *mitis* was isolated from a family member aged 3 years old in Jerusalem. Ribotyping was performed and the two isolates were found to be indistinguishable. Preliminary findings indicated that the ribotype was uncommon and had not previously been observed.

This case report indicated that disease transmission does not necessarily occur as an isolated event in one country but may have implications for spread to other countries. Frequent travel between Russia and Israel could be a factor of both these cases. It should also be noted that, in both case, the boys had received appropriate age-specific vaccination that protected them from contracting the severe form of the disease. An important conclusion is that all travellers should ensure they receive the full primary diphtheria vaccination and booster doses every ten years.

A 5.14: Molecular-biological monitoring of *Corynebacterium diphtheriae* circulation in Belarus

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Phenotyping and genotyping were performed on strains of *Corynebacterium diphtheriae* that were isolated in Belarus between 1996 and 2000, during a period of sustained disease decline. The purpose of this typing was to obtain characteristics of these strains, especially the epidemic clone. Biotyping and toxigenicity testing using the Elek test and PCR were carried out on 2002 isolates of *C. diphtheriae* from diphtheria patients (305 strains), tonsillitis patients (890), close contacts (347), and asymptomatic carriers (460). From 1997, there was a twofold decrease in the ratio of toxigenic to non-toxigenic *C. diphtheriae* strains. In 1996, the tox⁺/tox⁻ ratio was 1:1 and, between 1997 and 2000, the ratio was 1:2. However, between 1997 and 2000 the *var gravis* biotype continued to prevail and it constituted 59% of isolates. As usual, toxigenic strains prevailed among *var gravis* and their proportion was 19 times higher compared with *var mitis*. Ribotyping, using two restriction enzymes *Bst*Ell and *Pvu*II, revealed 18 ribotypes patterns amongst 240 *C. diphtheriae* (172 *var gravis*, 67 *var mitis* and one *var belfanti*). The majority of isolates belonged to the epidemic clone of ribotypes Sankt-Petersburg (26%) and Rossiya (27%). The ribotypes Cluj, Moskva, Otchakov, Lyon, and New constituted 13%, 12%, 8%, 5%, 4% isolates, respectively. The remaining 11 ribotypes represented 6% of the isolates. The connection of the distinct ribotypes with the source of *C. diphtheriae* strains isolation was established. Epidemic ribotypes Sankt-Petersburg and Rossiya were most common among diphtheria patients (72%, 116 of 161 cases), but were rarely reported among tonsillitis patients (12%, 6 of 51), close contacts (21%, 3 of 14), and asymptomatic carriers (7%, 1 of 14). Among imported cases ribotype Sankt-Petersburg dominated as well (4 of 5). Non-epidemic ribotypes were detected from four sources of *C. diphtheriae* isolation with various frequency. Strains of two ribotypes, Otchakov and Lyon (10% and 6%, respectively), were nearly always isolated from diphtheria patients. Among tonsillitis patients and close contacts, two ribotypes, Moskva (39% and 29%) and Cluj (29% and 43%) prevailed. Among carriers, 79% of strains were referred to ribotype Cluj. Eleven other ribotypes rarely occurred among diphtheria patients (9%), tonsillitis patients (18%), and in carriers (7%). The comparison of the genotypic and phenotypic features of *C. diphtheriae* demonstrated an absence of a clear correlation between them. Ribotypes Sankt-Petersburg, Rossiya, and Lyon were mainly toxigenic strains of biotype *var gravis*, the ribotype Moskva mainly represented non-toxigenic strains of *var mitis*, containing the fragment of A-tox-gene. However, ribotype Cluj included non-toxigenic strains (tox PCR)

of three biotypes (*var gravis*, *var mitis*, *var belfanti*). It is possible to identify the reemerging genotypes and the elimination of the epidemic clone, and to determine their role in the epidemiology of diphtheria by means of molecular epidemiological investigations.