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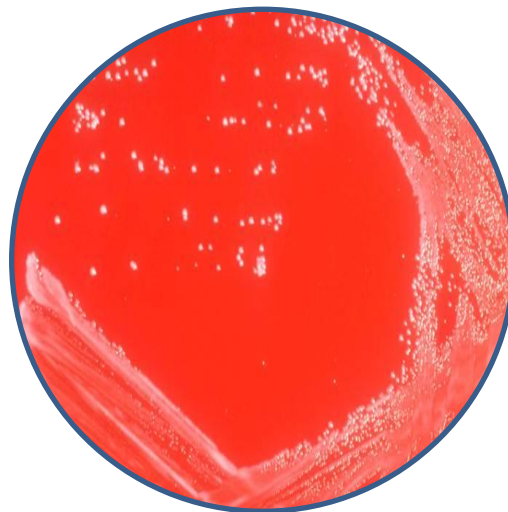REGIONAL OFFICE FOR **Europe**

WORKSHOP ON
LABORATORY DIAGNOSIS OF DIPHTHERIA

Report

11–13 October 2017

University of Cyprus

Nicosia, Cyprus

Abstract

The workshop on laboratory diagnosis of diphtheria was a collaborative effort by the WHO Collaborating Centre for Reference and Research on Diphtheria and Streptococcal Infections, Public Health England, United Kingdom and the WHO Regional Office for Europe. It was hosted by the Medical School of the University of Cyprus, Cyprus, and was attended by 12 laboratory personnel nominated by 11 newly independent states: one each from Armenia, Azerbaijan, Belarus, Georgia, Kazakhstan, Kyrgyzstan, the Republic of Moldova, the Russian Federation, Tajikistan and Uzbekistan and two from Ukraine. The workshop served to increase awareness about corynebacteria infections among participating laboratory personnel and equipped them with skills to laboratory confirm the disease, and to identify toxigenic strains and types of corynebacteria.

Keywords

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Cover photo: typical C. diphtheriae growth on blood agar (courtesy of the WHO Collaborating Centre for Reference and Research on Diphtheria and Streptococcal Infections, Public Health England)

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Abbreviations

EDSN	European Diphtheria Surveillance Network
MALDI	matrix-assisted laser desorption/ionization
MALDI - TOF MS	matrix-assisted laser desorption/ionization/ionization-time of flight mass spectrometry
qPCR	quantitative polymerase chain reaction
PCR	polymerase chain reaction

Acknowledgments

1. Executive summary

Following the massive re-emergence of diphtheria in the newly independent states of the former Soviet Union in the 1990s, the disease has become rare in the WHO European Region. Nonetheless, diphtheria and related infections caused by toxigenic strains continue to be reported and play a role as a lethal resurgent infectious disease. The rarity of cases and the expense and complexity associated with laboratory diagnosis led many countries in the Region to cease screening throat specimens; therefore, expertise and recognition of the organism declined. Consequently, in many countries laboratory expertise has declined. Without the possibility to microbiologically diagnose the disease, appropriate public health management cannot be effected. Diphtheria has the potential to re-emerge in areas where population immunity through vaccination is not maintained at high levels. Therefore, both clinicians and laboratory personnel should maintain a high index of suspicion in patients presenting with signs and symptoms of respiratory or cutaneous diphtheria particularly after being in countries endemic for the disease. The workshop served to increase awareness for corynebacteria infections among participating laboratory personnel and equipped them with skills to laboratory confirm the disease, and to identify toxigenic strains and types of corynebacteria.

2. Aim and objectives

The main aim of the workshop was to strengthen the participants' skills and to build capacity in the laboratory diagnosis of diphtheria. The expected outcome of the workshop was to establish a formal coordinated approach to strengthen diphtheria laboratory surveillance.

The main objectives of the workshop were:

1. to update microbiological focal points on the laboratory capabilities and needs (including quality assurance) of laboratories conducting diphtheria diagnostics;

2. to establish specialized methods for laboratory diagnosis of diphtheria and to teach participants the isolation and identification of the causative organisms directly from clinical specimens;

3. to identify the immediate and long-term needs (over the following 12 months) in terms of reagents and diagnostic materials required for laboratory diagnosis of diphtheria;

4. to undertake detailed discussions on aspects of microbiological diagnostics for diphtheria;

5. to provide participants with theoretical and practical information on epidemiological typing methods for *Corynebacterium diphtheriae* and other potentially toxigenic corynebacteria;

6. to discuss the current status of diphtheria and the immediate and long-term needs among countries represented at the workshop; and

7. to review the availability and quality of diphtheria antitoxin, and antibiotics and their distribution (cold-chain etc.).

3. Background

Significant gaps in the capacity to diagnose diphtheria exist in a number of Member States of the WHO European Region. This finding was based on the results of a gap analysis on securing diphtheria diagnostic capacity in 30 Member States in the European Union/European Economic Area[1] and preliminary results of the same analysis for 11 newly independent states. The largest gaps relate to laboratory training and to surveillance for all three potentially toxigenic diphtheria pathogens namely *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*.

To help address the gaps identified, the WHO Regional Office for Europe and the WHO Collaborating Centre for Reference and Research on Diphtheria and Streptococcal Infections conducted a training workshop on laboratory diagnosis of diphtheria in Nicosia, Cyprus, on 11–13 October 2017. Professor Nikolas Pavlides, Professor Androulla Efstratiou and Dr Mark Muscat opened the workshop and welcomed the participants. The workshop programme is in Annex 1 and a bibliography is in Annex 2. National experts responsible for laboratory diagnosis of diphtheria in 12 of the European Region's newly independent states were invited to participate. Of these, 11 Member States were represented at the workshop (see Annex 3).

The rapporteurs were Dr Muscat and Professor Efstratiou.

4. Structure of the workshop

The morning of the first day was structured as plenary sessions to inform about and to discuss the current epidemiological status of diphtheria and the challenges that countries with low or undetected incidence have in performing laboratory diagnosis of diphtheria. Laboratory training with hands-on learning started in the afternoon of the first day and continued until the afternoon of the third day. The workshop was conducted in Russian and English.

The workshop comprised interactive lectures and laboratory sessions predominantly with hands-on practical learning. Participants received a laboratory manual for the practical sessions and were taught:

- preparation of specialized media;
- processing of primary throat swab cultures;
- selection of colonies and colony morphology; screening tests to detect pyrazinamidase and cystinase activity; biochemical identification tests by conventional and commercial methods; and
- preparation of the Elek test for toxigenicity (conventional and modified); and polymerase chain reaction (PCR) to detect the toxin gene.

The workshop allowed for discussions on:

- antimicrobial susceptibility testing
- novel molecular assays for diphtheria diagnostics
- quality assurance for diphtheria diagnostics
- a proposal for a WHO Global Laboratory Diphtheria Network.

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[1] European Centre for Disease Prevention and Control. Gap analysis on securing diphtheria diagnostic capacity and diphtheria antitoxin availability in the EU/EEA. Stockholm: ECDC; 2017.

5. General epidemiology of diphtheria

Prior to the widespread use of diphtheria immunization, the disease was a major cause of death among children. The last largest outbreak in recent years took place in the Russian Federation in 1990. More than 115 000 cases and 3000 deaths were reported from 1990 to 1997.[2] Diphtheria is now considered uncommon in the European Region. Nevertheless, sporadic cases, sometimes resulting in death, continue to be reported. In 2015–2016, there were three case reports of fatal diphtheria, two of which were of unvaccinated children.[3] By contrast, the disease remains a serious public health problem in some countries of South-East Asia, South America and Africa.

Diphtheria has the potential to re-emerge in areas where population immunity through vaccination is not maintained at high levels. For 2015, six countries in the European Region reported <90% coverage with the three-dose primary series of diphtheria-containing vaccine. These included five countries that reported a 2–5% decline in coverage over the previous year. Suboptimum and declining coverage rates reported by these countries, as well as the general lack of availability of diphtheria antitoxin, have raised concern.

6. Laboratory support

The WHO Collaborating Centre for Reference and Research on Diphtheria and Streptococcal Infections, housed under the auspices of Public Health England, works closely with centres from across the globe. The WHO Collaborating Centre has considerable expertise in diagnostics, molecular epidemiological typing, surveillance studies, design and execution of external quality assurance studies, laboratory workshops and symposia within this specialized field.

The WHO Collaborating Centre was an essential component of the *European Laboratory Working Group on Diphtheria* that was established at the initiative of the Regional Office in 1993 as a result of the epidemic situation in eastern Europe to help strengthen the diphtheria diagnostic capabilities of many countries in the Region. The Working Group was later succeeded by the *European Diphtheria Surveillance Network* (EDSN) that was established in 2010 in collaboration with the European Centre for Disease Prevention and Control. EDSN comprises the nominated epidemiologists and laboratory experts for diphtheria from 30 countries of the Region. Its purpose is to establish a system of expertise to prevent diphtheria and to strengthen and to harmonize laboratory capacity at national level. The WHO Collaborating Centre organized and distributed the last external quality assessment study for diphtheria diagnostics in 2013.

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[2] Markina SS, Maksimova NM, Vitek CR, Bogatyreva EY, Monisov AA. Diphtheria in the Russian Federation in the 1990s. J Infect Dis. 2000; 181(Suppl.1):S27–34.

[3] Gaps found in the Region's capacity to diagnose diphtheria. Copenhagen: WHO Regional Office for Europe; 21 July 2017 (http://www.euro.who.int/en/health-topics/disease-prevention/vaccines-and-immunization/news/news/2017/07/gaps-found-in-the-regions-capacity-to-diagnose-diphtheria, accessed 15 February 2018).

6.1 Development of the manual on laboratory diagnosis of diphtheria

At the request of the WHO Regional Office for Europe, the manual on laboratory diagnosis that was originally published in 1981 was rewritten and published in 1994. Not surprisingly, the overall contemporary approach to laboratory diagnosis was not very different from that used in earlier years. In 1999, the then Public Health Laboratory Service updated the 1994 guidance for laboratory diagnosis, primarily in the collection and transportation of specimens, primary culture, microscopy, screening tests, biotyping and specialized reference centres, antimicrobial susceptibility testing, serological immunity assays, molecular diagnostic PCR-based assays and epidemiological typing. The 1994 manual has been extensively revised to take into account the developments within the field and the changing epidemiology of these infections. The manual will be published in 2018.

7. Microbiology and the laboratory diagnosis of diphtheria

Respiratory or cutaneous diphtheria is caused by toxigenic strains of *C. diphtheriae* and *C. ulcerans*, and, rarely, *C. pseudotuberculosis*. *C. diphtheriae* is a non-sporing, non-encapsulated and non-motile Gram-positive bacillus. Four biovars of *C. diphtheriae* can be distinguished biochemically: gravis, intermedius, mitis and belfanti. Both *C. diphtheriae* and *C. ulcerans* can produce an exotoxin that causes local tissue necrosis and, when absorbed into the bloodstream, causes toxaemia and systemic complications including paralysis due to demyelinating peripheral neuritis and cardiac failure due to myocarditis. The structural gene of the diphtheria toxin, *tox*, is carried by a family of corynebacteriophages. The toxin is a 535 amino-acid 58 kDa exotoxin whose active form consists of two polypeptide chains linked by a disulphide bond. The clinical and epidemiological significance of non-toxigenic *C. diphtheriae and C. ulcerans* is unclear.

Laboratory diagnosis is by culture of an isolate of *C. diphtheriae*, *C. ulcerans* or *C. pseudotuberculosis* in a clinical laboratory. The common detection methods in use in most laboratories are microbiological culture on standard agar (or tellurite-containing media) with Gram stain of a suspicious colony. Further identification of catalase positive, Gram-positive coryneforms may be performed by conventional biochemical testing. Increasingly, many laboratories also use phenotypic methods such as matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). All of these methods can have good specificity, but the confirmation of identification and the determination of toxigenic strains usually require submission to the national reference laboratory. Conventionally the identification of a toxigenic strain is usually performed using the Elek test to detect toxin expression. The phenotypic Elek test takes ≥24 hours and confirmation of species identity of submitted isolates by traditional phenotypic methods can take ≥48 hours. PCR assays are available and can identify and detect the tox-bearing gene or nontox-bearing *C. diphtheriae*, *C. ulcerans*/*C. pseudotuberculosis* in DNA extracts from isolates within 4 hours (Fig. 1).

Swabs (nasopharyngeal, throat, wound or skin lesions) should be obtained for culture before starting treatment. Where a pseudomembrane or membrane is present, if possible, swabs should be taken from underneath the pseudomembrane or a piece of the membrane should be removed. If antibiotics have already been commenced, specimens for culture should still be taken. Clinicians should alert the local laboratory that diphtheria is suspected.

Fig. 1. Algorithm for the laboratory diagnosis of diphtheria
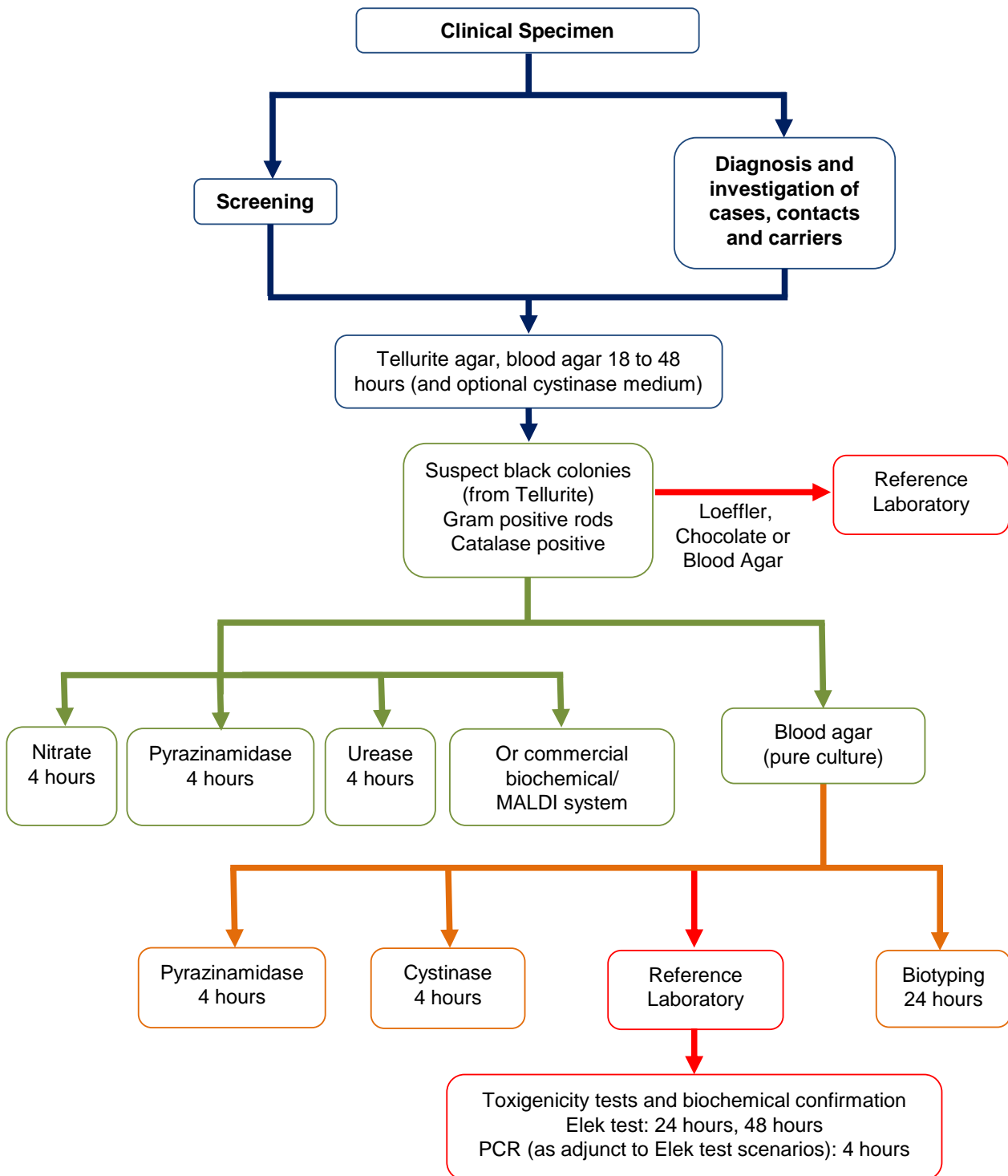
*A*ll isolates of potentially toxigenic corynebacteria (*C. diphtheriae*, *C. ulcerans* or *C. pseudotuberculosis*) should ideally be submitted to a reference/specialist laboratory for confirmation of identification and **toxigenicity testing**. Identification/confirmation and toxigenicity testing can be performed initially by conventional or real-time PCR (qPCR)[4] on a DNA extract of the isolate. Isolates which are qPCR positive for the toxin gene (tox) must be tested by the Elek test for toxin expression.

Although *C. diphtheriae, C. ulcerans* and *C. pseudotuberculosis* toxin gene PCR positive results will be confirmed by the Elek test, **a toxin gene PCR positive result should be acted upon without waiting for the Elek result.** A toxin gene PCR negative result is final and no further toxigenicity testing should be undertaken on these isolates.

Rarely, isolates of *C. diphtheriae* are *tox* gene positive by PCR but do not express toxin; i.e., when tested in the Elek test they will give a negative result. These are very rare in the United Kingdom. They will not cause diphtheria and so patients are not treated with antitoxin. If detected in symptomatic patients or asymptomatic carriers, they should, however, be eliminated using antibiotics in the same way as fully toxigenic strains. However, their presence/distribution in other global regions is unknown. It is important that awareness of these atypical isolates is highlighted.

8. Conclusions

The types of infections caused by *C. diphtheriae* and, more recently, *C. ulcerans* have changed over the last 2–3 decades. The emergence of toxigenic *C. ulcerans* and its possible correlation with domestic animals and the significance of non-toxigenic strains causing systemic disease have underlined the need for screening. As diphtheria resurged in the European Region, methodologies were reviewed and revived in many laboratories after having been discontinued years ago. However, more recently, as a consequence of the decline of the disease in the European Region, there have been mixed views about the need for laboratories to screen routinely for potentially toxigenic corynebacteria. The rarity of cases, and the expense and complexity associated with laboratory diagnosis led many countries in the Region to cease screening throat specimens; therefore, expertise and recognition of the organism declined. Consequently, in many countries the need to perform laboratory screening has reversed once again and many countries have discontinued routine screening. This is cause for concern and has considerable public health implications, particularly since toxigenic strains still persist and clinical diphtheria cases are still reported from countries within the European Region and beyond. Therefore, laboratory diagnostics and surveillance require strengthening.

The major role of the laboratory is the provision of simple, rapid and reliable methods to assist clinicians in confirming a clinical diagnosis. It is, however, sometimes often difficult to diagnose diphtheria clinically, particularly in countries where the disease is rarely seen. Diphtheria can often be confused with other presentations such as severe streptococcal sore throat, Vincent's angina or glandular fever. Therefore, accurate microbiological diagnosis is crucial and is always regarded as being complementary to clinical diagnosis. The laboratory also aids the clinician by eliminating suspected cases or contacts from further clinical investigation, thus avoiding unnecessary treatment or control measures.

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[4] Real-time PCR is also known as a quantitative PCR (qPCR).

Lastly, it is essential that each country has a reliable case reporting system; reporting toxigenic isolates is mandatory in the European Region and the United States of America. This is in accordance with the WHO and European Union case definitions; thus, all toxigenic isolates of *C. diphtheriae* and *C. ulcerans* must be reported. It is also important for laboratories to liaise closely with microbiologists and epidemiologists of the national reference laboratory. Below are a set of recommendations emanating from the discussions held during the workshop.

9. Recommendations

1. Develop an updated WHO manual for microbiological diagnosis of diphtheria (in English and Russian).

2. Strengthen laboratory capacities to ensure rapid primary diagnosis.

3. Identify funds for materials that enable laboratories to operate effectively.

4. Develop an external quality assurance scheme for diphtheria diagnostics.

5. Have at least one laboratory in each country assigned as the national reference laboratory and maintain capacity for toxigenicity testing.

6. Provide workshops and training in specimen taking and transportation, and clinical and laboratory diagnosis for staff, in collaboration with the WHO Collaborating Centre for Reference and Research on Diphtheria and Streptococcal Infections and the Regional Office.

7. Improve laboratory capacity in specific areas.

8. Re-establish a network of national focal points responsible for laboratory and epidemiological aspects of diphtheria.

9. Consider undertaking prevalence studies in specific situations (e.g. low vaccination coverage areas).

Annex 1. Programme

DAY 1: Wednesday 11 October 2017

Registration

Introduction and welcome

       Professor Nikolas Pavlides

       Professor Androulla Efstratiou

       Dr Mark Muscat

Introduction of the participants

Diphtheria: the disease and its epidemiology

       Dr Mark Muscat

Laboratory diagnosis of diphtheria and related infections

       Professor Androulla Efstratiou

Molecular diagnostics and typing of pathogenic *Corynebacterium diphtheriae* and *C. ulcerans*

       Dr Baharak Afshar

PRACTICAL SESSION 1

In Teaching Laboratory

Primary cultures and toxigenicity tests

- Preparation of conventional and modified Elek plates
- Examination of primary throat swab cultures (C1, H1, T1): colony morphology; selection of colonies onto blood agar, Hoyle's tellurite and Tinsdale agar
- Inoculation of Elek plates (from pure isolates, A, B)

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DAY 2: Thursday 12 October 2017

PRACTICAL SESSION 2

- Reading 24-hour toxigenicity tests on strains C and D
- Examination of subcultures from primary plates (blood agar, Hoyle's plate cultures): (A, B)
- Screening/identification tests from subcultures of primary plates (4h and 24h tests)(Conventional tests/API/ ROSCO)
- Reading 24-hour screening test from yesterday (Tinsdale)
- API Coryne test to be set up

DAY 2: Thursday 12 October 2017 *(contd)*

- DNA extraction of cultures C and D for PCR
- Demonstration and preparation of PCR assay
- Reading of 4-hour screening test results from tests prepared in the morning
- PCR assay preparation

DISCUSSION

- Novel molecular diagnostics for diphtheria – RTPCR
- Antimicrobial susceptibility testing: discussion of methodology
- Serological methods

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DAY 3: Friday 13 October 2017

PRACTICAL SESSION 3

- Final Results of throat cultures (API reading and other tests)
- Reading 48-hour toxigenicity results for C and D
- Preparation of E gels for PCR assay
- Preparation and loading of PCR products on agarose gel (C and D)
- Visualization of PCR fragments on gel (A, B)

DISCUSSION

- Forming a national diphtheria network and increase regional activities
- Achieving a standard level of diphtheria diagnostics for hospital laboratories
- Screening in diagnostic laboratories
- Quality assurance in the newly independent states
- Difficulties/problems in laboratory diagnosis of diphtheria
- Training and exchange of staff
- Any other business

Concluding remarks and presentation of certificates

Professor Androulla Efstratiou and Dr Mark Muscat

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