TECHNICAL REPORT

External quality assessment scheme for *Bordetella pertussis* antimicrobial susceptibility testing, 2022

On behalf of ERLNPert-Net surveillance network

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ERLNPert-Net surveillance network

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Contents

Abbreviations

AST	Antimicrobial Susceptibility Testing
AZT	Azithromycin
COVID-19	Coronavirus disease 2019
DNA	Deoxyribonucleic Acid
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EQA	External Quality Assessment
ERY	Erythromycin
ERLNPert-Net	European Reference Laboratory Network of pertussis experts Consortium funded by ECDC for this programme
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EUPert-LabNet	Name of former consortium of European Pertussis Laboratory Surveillance Network funded by ECDC
MIC	Minimum Inhibition Concentration
NRL	National Reference Laboratory
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SXT	Trimethoprim/Sulfamethoxazole
USA	United States of America
UTU	University of Turku (Turku, Finland)
WGS	Whole Genome Sequencing

Executive summary

Pertussis (whooping cough) is an acute bacterial infection usually caused by *Bordetella pertussis*, which can affect people of all ages [1-2]. Macrolides [erythromycin (ERY) and azithromycin (AZT)] are the recommended drugs to treat *B. pertussis* infection in many countries [2, 3]. To date, macrolide resistant *B. pertussis* isolates have mainly been found in China, but such isolates have also been found sporadically in Europe, the Middle East and in North and South America [4-10]. In a previous ECDC-funded (ECDC/2015/009) European Pertussis Laboratory Surveillance Network (EUPert-LabNet) project, training for antimicrobial susceptibility testing was provided to reference laboratories from a number of European countries.

This report presents the results of the first external quality assessment (EQA) scheme for *B. pertussis* antimicrobial susceptibility testing by ECDC as part of the European Reference Laboratory Network for Pertussis (ERLNPert-Net) consortium. Sixteen laboratories in 16 countries (out of 28 invited, 57.1%) participated in the EQA scheme, however results were only obtained from 13 of the 16 (81.3%) laboratories as three (18.9%) were unable to perform testing. Participating laboratories were from or working on behalf of 16 EU/EEA countries.

The specific aims of this *B. pertussis* AST EQA scheme were to: evaluate the ability of participating laboratories to perform genotypic antimicrobial susceptibility testing of *B. pertussis* based on nucleic acid analysis; to assess differences in methodology, interpretation and reporting of results; to identify future training needs; and to assist with the establishment of 'best practice' in current assays, interpretation and reporting.

In this EQA, the test panel included 11 samples. These samples contained DNA extracted from macrolide sensitive and resistant *B. pertussis* (in different concentrations), DNA from clinical samples which tested negative for *B. pertussis* and PBS (as no DNA sample). The EQA scheme was designed for genotypic identification of sensitive/resistant *B. pertussis* (PCR/Sanger sequencing/WGS based). This strategy was feasible as the only known mechanism for *B. pertussis* macrolide resistance is associated with a single point mutation (A2047G) within its 23S rRNA gene.

For identification of macrolide resistant *B. pertussis*, different protocols were used. Of the 13 laboratories that provided the results, 11 (84.6%) used block-based PCR, one (7.7%) used qPCR and 2 (15.4%) used Sanger sequencing and two (15.4%) used whole genome sequencing (WGS). Block-based PCR was used solely by nine (69.2%) laboratories and Sanger sequencing by two (15.4%) laboratories. The other two (15.4%) laboratories used multiple techniques.

Results for the first EQA on antimicrobial susceptibility testing of macrolide sensitive/resistant *B. pertussis* were encouraging as nine (69.2%) laboratories had only one or no samples incorrectly reported: five out of 13 (38.5%) laboratories scored 11/11 (100%, intended results) correctly and four (30.8%) laboratories scored 10/11 correctly. The remaining four laboratories had five to nine panel samples correctly identified. The results indicate that low DNA concentration was a major obstacle for application of the WGS approach for identification of resistant/sensitive *B. pertussis* in EQA samples, and that PCR based methods provided better results.

Pertussis national reference laboratories (NRLs) should be able to correctly perform antimicrobial susceptibility testing (AST) based on genotyping and phenotyping. This EQA round highlighted training needs in the identification of macrolide resistant *B. pertussis* by DNA based approaches. Furthermore, guidelines, such as proper test controls, importance of DNA concentration suitable for detection with different methods and how to interpret the results would provide added value.

1. Background

The European Centre for Disease Prevention and Control is a European Union agency with a mandate to operate surveillance networks and to identify, assess and communicate current and emerging threats to human health from communicable diseases. As part of its mission, ECDC shall 'foster the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents which may threaten public health. The ECDC shall maintain and extend such cooperation and support the implementation of quality assessment schemes' (Article 5.3, EC 851/2004).

External quality assessment (EQA) rounds are an integral part of a quality management system. They evaluate the performance of laboratories by an outside agency on material that is supplied especially for this purpose. ECDC organises a series of EQAs for EU/EEA countries. A list of completed EQAs is provided in Annex 1. In addition, some non-EU/EEA countries are also involved in these EQA activities. The aim of EQAs are to identify areas for improvement in laboratory diagnostic and reference capacities relevant to the surveillance of the diseases listed in Decision No. 2119/98/EC 2, and to ensure comparability of results between laboratories from all EU/EEA countries.

The main purposes of EQA schemes include:

- Assessment of the general standard of performance ('state of the art');
- Assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration);
- Evaluation of individual laboratory performance;
- Identification and justification of problem areas;
- Providing continuing education;
- Identification of needs for training activities.

Pertussis (whooping cough) is an acute bacterial infection usually caused by *Bordetella pertussis*, which can affect people of all ages. Newborns and unvaccinated infants are the most vulnerable groups with the highest rates of morbidity and mortality, whilst older children and adults usually display milder symptoms [1-2]. Similar but less severe illnesses may be caused by *Bordetella parapertussis* and *Bordetella holmesii,* however vaccination with the current pertussis vaccines does not protect against these two species. Older age groups are an important reservoir and source of infection for infants. Increases in awareness of pertussis infections and reported cases in several countries have highlighted the need for good laboratory methods for the detection, identification and characterisation of clinical infections caused by *B. pertussis* and other *Bordetella* species.

Macrolides [erythromycin (ERY) and azithromycin (AZT)] are the recommended drugs to treat *B. pertussis* infection in many countries [2, 3]. To date, macrolide resistant *B. pertussis* isolates have mainly been found in China, but such isolates have also been found sporadically in Europe, the Middle East and in North and South America [4-10]. The frequency of these isolates has increased in many parts of China. According to Chinese studies performed in Beijing (2013–2014), Zhejiang province (2016) and Shanghai (2016–2017), frequencies of macrolide resistant *B. pertussis* varied from 60% to 92%, which is alarming [11, 12]. Recently, macrolide resistant *B. pertussis* isolates have been observed in Japan and Vietnam [13, 14]. If macrolides cannot be used, the option is sulphamethoxazole/trimethoprim. However, due to potential side effects, it is not recommended for the treatment of infants <2 months of age. Lately, piperacillin and cefoperazone-sulbactam have shown high efficacy to eliminate *B. pertussis* both in vitro and in vivo. Although more clinical data are needed, these two antibiotics provide good alternative treatment in infants [15, 16].

So far, the only mechanism identified to cause macrolide resistance has been a point mutation, changing nucleotide A to G, at position 2047 (A2047G) in the domain V of the 23S rRNA gene of *B. pertussis*, as described previously by many studies [3, 7, 10, 17]. However, it remains to be shown if other mutations or molecular changes in the genome confer macrolide resistance. Therefore, phenotypic culture-based antimicrobial susceptibility testing (AST) is still the golden standard for the determination of macrolide resistance. It has been shown that almost all resistant *B. pertussis* isolates have high minimum inhibitory concentrations (MIC) to macrolides (>256 µg/mL to both ERY and AZT) indicating profound resistance, whereas sensitive isolates show no tolerance against the macrolides (<0.250 µg/mL to both ERY and AZT) [3, 7, 12, 18]. Crucially, there are no defined cut-offs from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) regarding macrolides and *B. pertussis*. However, if these strains would expand to Europe in high numbers, EU/EEA Member States should have the capacity and capability to rapidly identify these isolates and be prepared to take appropriate public health action.

Methods to detect macrolide resistance have been described in detail. Wang et.al developed an allele specific PCR for rapid detection of the A2047G mutation in the 23S rRNA gene, the primers used are presented in Table 3 and the complete protocol is available [9]. PCR-based (partial) Sanger sequencing of 23S rRNA gene or whole genome sequencing (WGS) are other options to detect these isolates, but WGS is not as practical for small-scale laboratories as PCR or PCR-based Sanger sequencing of the 23S rRNA.

In a previous ECDC-funded (ECDC/2015/009) European Pertussis Laboratory Surveillance Network (EUPert-LabNet) project, training for antimicrobial susceptibility testing was provided to reference laboratories from a number of European countries.

EQAs are critical components in assessing laboratory performance. As part of the Coordination of the European Reference Laboratory Network for Pertussis (ERLNPert-Net), Framework Service Contract (FWC), ECDC/2019/023, University of Turku (UTU) was responsible to organize work package 2: EQA scheme for detection of susceptibility testing of *B. pertussis* isolates among the NRLs in Member States, Iceland, Liechtenstein and Norway. This report summarises the layout and results of this EQA round.

2. Introduction

Pertussis is endemic worldwide and is an important cause of morbidity and mortality, particularly in newborns and unvaccinated infants. Despite long-standing vaccination programmes, pertussis remains an important public health issue. To ensure national control strategies, high quality laboratory methodology and epidemiological information are required for the diagnosis of cases as well as to ensure appropriate treatment, prevention, vaccine effectiveness, management and surveillance of the disease.

Previous ECDC *Bordetella* EQA schemes have addressed different laboratory methods for *Bordetella* species identification and surveillance of *B. pertussis* circulating isolates using phenotypic and genotypic methods [19]; *B. pertussis* serology [20, 21] and detection of *B. pertussis* nucleic acids by PCR [22].

Within the last 10 years, *B. pertussis* isolates resistant to macrolides have appeared globally with a high notification rate in different provinces of China [9, 12, 23-24]. MIC testing is the gold standard but it is not always possible to perform isolates lacking due to reduced culturing of clinical specimens . Therefore, approaches based on PCR and Sanger sequencing are widely used to identify the only known mechanism so far (A2047G mutation in the 23S rRNA gene) to cause macrolide resistance [17]. Detection of macrolide resistance is a public health priority in pertussis surveillance and therefore the ability to detect *B. pertussis* macrolide resistance based on MIC or PCR/Sanger sequencing is of high importance for effective surveillance activities.

EQA schemes are important for accurate identification and assessment of laboratory performance. This current programme is designed to assist the assessment, development and standardisation of techniques for the characterisation of the antimicrobial susceptibility testing (AST) of *B. pertussis* isolates. Furthermore, MIC testing is more commonly used by diagnostic laboratories in Europe, so preparedness for PCR-based detection should be established. The design of the EQA was intended to cover the different capacity and access to technologies across participating laboratories. This EQA scheme aimed for DNA-based detection of macrolide resistant/sensitive *B. pertussis*. Laboratories were encouraged to use their own methodology, but we provided two possible protocols to be used, which have been used before in the Finnish National Reference Laboratory (NRL) for Pertussis and Diphtheria (UTU, Turku, Finland) and were previously published [10, 24].

EQA schemes enable the performance of the NRLs from each EU/EEA Member State to be independently assessed. They also lead to recommendations for improvements to methodologies and identify areas for further training.

The specific aims of this *B. pertussis* AST EQA scheme were to:

- evaluate the ability of participating laboratories to perform genotypic antimicrobial susceptibility testing of *B. pertussis* based on nucleic acid analysis;
- assess differences in methodology, interpretation and reporting of results;
- identify training needs;
- assist with the establishment of 'best practice' in current assays, interpretation and reporting.

3. Materials and methods

3.1 Organisation

The ERLNPert-Net *B. pertussis* antimicrobial susceptibility testing EQA was organised by the Finnish NRL for Pertussis and Diphtheria [University of Turku (UTU), Institute of Biomedicine], Turku, Finland. It was intended for NRLs in EU Member States and EEA countries (Iceland, Liechtenstein and Norway). Invitations were sent by e-mail on 21 February 2022 to designated ECDC Operational Contact Points for Microbiology of Pertussis, which included laboratory experts in 28 countries. In total, 16 laboratories participated, 15 of which were EU countries and one an EEA country (Iceland) (Annex 2).

3.2 Selection of the panel

EQA panel consisted of extracted DNA from clinical isolates of *B. pertussis* (extracted, quality tested and divided into separate panels at the UTU). These isolates were selected from: (i) a Chinese *B. pertussis* isolate NAP-12-30 kindly provided by Dr. Zengguo Wang, Xi'an Children's Hospital, Xi'an, China, (ii) the UTU *B. pertussis* culture collection. The panel was designed to include DNA from macrolide sensitive and resistant *B. pertussis* isolates. In addition, extracted DNA from clinical respiratory samples negative to *B. pertussis*, phosphate buffered saline (PBS), and sensitive/resistant control DNA were included in the final EQA panel (Table 1).

3.3 Distribution of panels and instructions

The panels of test and control DNA were prepared, packed according to local regulations and shipped (on dry ice) on 19 April 2022. They were successfully delivered to 15 laboratories. However, for one laboratory the panel was lost by the courier company and another panel was sent on 2 May 2022. Since only DNA and no infectious pathogens were shipped, the incident did not pose a public health risk. Eventually, all packages arrived in good condition (frozen samples and intact packaging) to participating laboratories (confirmations received from each individual laboratory). Detailed instructions and reply forms were emailed to participants (Annex 3).

Table 1. Characteristics of the DNAs included in the ERLNPert-Net *B. pertussis* antimicrobial susceptibility testing EQA panel, including three strains, clinical samples and controls

Original reference no.	Sensitive / Resistant	Description
PRCB866	Sensitive	DNA from Clinical Finnish isolate
PRCB874	Sensitive	DNA from Clinical Finnish isolate
NAP-12-30	Resistant	DNA from Clinical Chinese isolate
Negative clinical pool	No *B. pertussis* DNA	DNA from pooled respiratory samples negative for *B. pertussis*
PBS	No DNA control	Phosphate buffered saline, no DNA included
Control DNAs		
ATCC9797	Sensitive	DNA from sensitive control strain
NAP-12-30	Resistant	DNA from resistant control strain

**NAP-12-30 has been shown to be highly resistant to macrolides [10].*

3.4 Testing

Participants were instructed to perform AST testing according to their standard methods, e.g. PCR, Sanger sequencing or whole-genome sequencing.

Participants were asked to determine whether the EQA panel samples contained DNA from macrolide resistant or sensitive B. pertussis. In addition, if no DNA was observed within the panel samples, that should be reported as no DNA or equivocal. As previously stated, we also provided two possible protocols to be used for the identification (Annex 4). The first protocol was based on block-based PCR and the second for real-time PCR using high resolution melting analysis adapted from the publications by Zhang Q et al. and Wang Z et al., respectively [10, 24].

A reporting sheet for the results was included with the instructions and sent to each participant by e-mail (MS Word document) to be returned by the deadline (Annex 4).

3.5 Data analysis

The intended results from the organising laboratory (Table 2) were used as a basis for the scoring. Participants were expected to:

- perform the laboratory and bioinformatic steps of genotypic AST of the *B. pertussis* isolates;
- determine the AST status of *B. pertussis* DNA included in the EQA panel using their own methodology or the provided methods;
- accurately interpret and report results depending on the methods used and results obtained;
- to code the presence of the A2047G mutation in the 23S rRNA gene as 'resistant' and its absence as 'sensitive'. Failure to report on this mutation was considered as incorrect result. Absence of genomic material was to be reported as 'no target DNA'.

Table 2. Intended results for ERLNPert-Net *B. pertussis* antimicrobial susceptibility testing EQA panel (February 2022)

EQA panel number	Original reference no.*	DNA concentration (ng/µl)	Intended result (Sensitive/resistant/no DNA)
EQA1	PRCB866	5ng/ul	Sensitive
EQA2	PRCB874	5ng/ul	Sensitive
EQA3	NAP-12-30	5ng/ul	Resistant
EQA4	NAP-12-30	1ng/ul	Resistant
EQA5	NAP-12-30	0.1ng/ul	Resistant
EQA6	NAP-12-30	0.01ng/ul	Resistant
EQA7	NAP-12-30	0.001ng/ul	Resistant
EQA8	NAP-12-30	0.0001ng/ul	Resistant
EQA9	Negative clinical sample pool	Original	No DNA / Equivocal
EQA10	Neg. pool + NAP-12-30	Original + 0.1ng/µl NAP-12-30	Resistant
EQA11	PBS	-	No DNA / Equivocal
Controls	**Original reference no.**	**DNA concentration (ng/µl)**	**Intended result (Sensitive/resistant/no DNA)**
S-control	ATCC9797	5ng/ul	Sensitive
R-control	NAP-12-30	5ng/ul	Resistant

**NAP-12-30 has been shown to be highly resistant to macrolides [10]*

4. Results

The samples included in the EQA panel consisted of resistant or sensitive *B. pertussis* DNA with versatile concentrations, ranging from 0.0001ng/µl to 5ng/µl. In addition, PBS and two pooled clinical samples including either resistant *B. pertussis* DNA or no *B. pertussis* DNA were included (Table 1).

AST was performed by block-based PCR only in nine (69.2%) laboratories and by WGS only in two (15.4%) laboratories. In total, block-based PCR was used by 11/13 (84.6%) laboratories, qPCR by 1/13 (7.7%) laboratories, WGS by 2/13 (15.4%) and Sanger sequencing by 2/13 (15.4%) laboratories. One laboratory (7.7%) used three methods and one laboratory (7.7%) two methods. Methods per laboratory are presented in Table 3.

Table 3. *Bordetella pertussis* antimicrobial susceptibility testing methods used, by laboratory

Method	Laboratory no.												
	1	2	4	5	7	8	9	11	12	13	14	15	16
Block-based PCR	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
qPCR	No	No	No	No	No	No	Yes	No	No	No	No	No	No
Sanger sequencing	No	No	No	No	No	No	Yes	No	Yes	No	No	No	No
Whole-Genome Sequencing	No	No	No	Yes	Yes	No	No	No	No	No	No	No	No
Other	No	No	No	No	No	No	No	No	No	No	No	No	No

4.1 Genotypic antimicrobial susceptibility testing

Thirteen out of the 16 participating laboratories submitted AST results. Laboratory numbers 3, 6 and 10 did not perform AST testing. Two of these laboratories had technical problems with laboratory instruments and were not able to perform testing. One laboratory did not perform the EQA as it needed to de-prioritise participation in the scheme due to other duties in response to the COVID-19 pandemic. Therefore these three laboratories are not included in the results analysis.

From the 13 laboratories where results were obtained, five identified all panels (100%) correctly and four laboratories had 10/11 panels (90.9%) correctly identified. The remaining four laboratories identified the following number of panels correctly: 9/11 (81.8%) correct, 7/11 (63.6%) correct, 6/11 (54.5%) correct, and 5/11 (45.5%) correct.

Samples EQA1 and EQA2 included 5ng/µl DNA from macrolide-sensitive *B. pertussis* isolates. These samples were correctly identified by 12 out of 13 laboratories. EQA samples 3-8 included diluted DNA (5ng/µl to 100ng/µl) from macrolide-resistant *B. pertussis* isolates. From these, EQA samples 3 and 4 were correctly identified by all participating laboratories, while results for EQA samples 5-8 had incorrect results reported by a small number of laboratories (Table 4). EQA9 contained DNA isolated from clinical samples which tested negative for *B. pertussis*. This sample was correctly reported by only 6 out of 13 laboratories (46.2%). EQA10 contained the same clinical extraction as EQA9, but was spiked with DNA extracted from resistant *B. pertussis*. This sample was correctly identified by 11 of 13 laboratories. EQA11 contained PBS and was correctly identified by all laboratories (excluding one, where no result was obtained for this sample). Results from this EQA round are presented in Table 4, and red colouration of a laboratory's results indicate a discrepancy with the intended results.

Table 4. Results of *B. pertussis* antimicrobial susceptibility testing

EQA panel number	Intended result (S/R/no DNA**)	Laboratory no.												
		1	2	4	5*	7	8	9	11	12	13	14	15	16
EQA1	Sensitive (S)	S	S	S	S	S	S	S	S	S	R	S	S	S
EQA2	Sensitive (S)	S	S	S	S	S	S	S	S	S	R	S	S	S
EQA3	Resistant (R)	R	R	R	R	R	R	R	R	R	R	R	R	R
EQA4	Resistant (R)	R	R	R	R	R	R	R	R	R	R	R	R	R
EQA5	Resistant (R)	R	R	R	R	no DNA	R	R	R	R	R	R	R	R
EQA6	Resistant (R)	R	R	R	-	no DNA	R	R	R	R	R	R	R	R
EQA7	Resistant (R)	R	R	R	-	no DNA	R	R	R	R	S	R	R	R
EQA8	Resistant (R)	R	R	R	-	no DNA	R	R	R	R	S	R	R	R
EQA9	No DNA / Equivocal	S	no DNA	no DNA	-	no DNA	S	no DNA	S	no DNA	S	S	R	no DNA
EQA10	Resistant (R)	R	R	R	-	R	R	R	R	R	R	S	R	R
EQA11	No DNA / Equivocal	no DNA	no DNA	no DNA	-	no DNA	no DNA	no DNA	no DNA	no DNA	no DNA	no DNA	no DNA	no DNA

** Laboratory no. 5 reported that DNA concentration was too low for WGS, no results were obtained for EQA6-EQA11.*
*** no DNA refers to no B. pertussis target DNA in the sample. Sample EQA9 did contain human DNA and may have contained DNA of other bacterial species that the methods may have reacted to.*

5. Discussion

Bordetella pertussis antimicrobial susceptibility testing

This is the first ECDC EQA to assess the antimicrobial susceptibility testing of laboratories to detect macrolide resistant or sensitive *B. pertussis*. The number of original participants was 16 (28 invitations sent), which reflects an interest in this topic among the EU/EEA pertussis reference laboratories.

Macrolides are the first-line drugs used to treat pertussis patients. Although macrolide resistant isolates are still very scarce in Europe, they are prevalent in China and are expanding to other countries as well [7, 9, 13, 14, 25]. If an isolate is identified to be macrolide resistant, alternative drugs for clinical and prophylactic treatments are needed. Some antibiotics like the widely used amoxicillin has been shown to be effective in vitro, but ineffective in vivo [26]. Although trimethoprim/sulfamethoxazole (SXT) is a valid option [3, 27], SXT is not suitable for infants less than two months of age [27]. In two novel studies, piperacillin and cefoperazone-sulbactam have been shown to be effective both in vitro and in vivo, providing good options for alternative treatment, although their suitability for young infants still needs to be better studied [15, 16].

Commonly, many laboratories use phenotypic MIC testing to identify antimicrobial resistance among *B. pertussis* and other bacteria. However, the only known mechanism behind macrolide resistance of *B. pertussis* is identified in the 23S rRNA gene as a single nucleotide point mutation (SNP) A2047G, and can therefore be directly identified from the bacterial DNA [7, 10, 17, 24]. Results of a block-based PCR assay (available within the same day) or WGS-based approaches (available within a few days), are more rapid than obtaining a pure culture and using MIC testing (can take up to ten days). Furthermore, the lack of performed *B. pertussis* culture makes MIC testing difficult. Use of PCR or Sanger sequencing based identification is not dependent on culture as only a fraction of DNA is needed to identify the SNP causing the resistance.

The results of this EQA were encouraging as nine laboratories (69.2%) had either 10/11 or all eleven EQA panel samples correctly identified. Most of the laboratories used block-based PCR to identify the SNP causing the resistance. Correct observations were obtained for the most diluted EQA panel sample (0.0001ng/µl) by 10/13 (76.9%) laboratories. This indicates that only a small fraction of the DNA is needed for correct identification and further indicates that direct typing from nasopharyngeal swab samples is possible. However, the 23S rRNA subunit is also present in other bacteria (e.g. *M. pneumoniae*), which contain similar sequences to *B. pertussis* and may lead to false results without confirmation that *B. pertussis* DNA is present [28]. Results from this EQA round further highlights this problem as the EQA panel sample 9 contained DNA from clinical samples tested negative for *B. pertussis,* but some laboratories reported a sensitive genotype and one laboratory reported a resistant genotype which could possibly point towards cross-/contamination in the laboratory processes. Laboratory contamination is a well recognised concern [29]. However, in this EQA round similar sequences to *B. pertussis* have probably been the reason behind the high percentage of false results for the EQA sample 9 among the participating laboratories, not a contamination within the laboratory facilities. To avoid false-positive results, *B. pertussis* specific identification is and was needed to exclude this possibility (similar to diagnostics). Another laboratory reported two different samples with macrolide-sensitive isolate DNA as resistant and also reported the two resistant isolates with the least amount of DNA as sensitive, which again is a cause for concern and warrants close examination of the sources of errors, such as accidential mix up of samples.

According to the results, the 11 laboratories using the PCR based approach obtained better results than the two performing WGS. This difference can be explained by the sensitivity of WGS as some samples did not contain enough DNA. Although WGS is highly discriminative, it is more suitable for samples containing a higher bacterial load, for outbreak investigations and strain surveillance from cultured isolates, rather than for clinical specimens containing only a small amount of the DNA of interest.

In summary, the increase of *B. pertussis* resistant to macrolides requires appropriate laboratory techniques for monitoring and surveillance purposes. As discussed previously, alternative antibiotics can be used to treat patients caused by macrolide resistant *B. pertussis* [15, 16, 28]. PCR or PCR-based Sanger sequencing are classic techniques performing DNA-based identification and have been shown to be effective tools for the surveillance of *B. pertussis* isolates [7, 10, 17, 24]. WGS is most effective for outbreak investigations to discriminate pathogenic isolates, but lacks sensitivity for samples containing a low amount of DNA. It is relatively expensive, lacks standardisation and is more time consuming than direct PCR typing from a clinical specimen [30].

Identification of training needs

The overall score of this EQA round was decent. However, we identified participating laboratories having difficulties with low concentrated DNA samples and with performing the tests. Some of the participating laboratories have previously received training on AST at the UTU. However, training was only offered for a small fraction of EU/EEA countries. It seems apparent that further training is required for selected laboratories and guidelines on how to identify macrolide resistant *B. pertussis* is needed in the EU/EEA.

Although invitations were sent to 28 EU/EAA countries, only 16 laboratories participated in this EQA round. This may indicate that the missing National Reference Laboratories (NRLs) do not have functional tests for DNA based AST and/or do not routinely perform these. However, it is important to note that this EQA round was carried out during the COVID-19 pandemic, and staff from many pertussis NRLs were involved in COVID-19 diagnostic and surveillance activities. One laboratory expressed interest in participating but in the end was not able to produce the results due to COVID-19 activities. It is possible that other invited laboratories did not have capacity to participate in this EQA due to COVID-19 activities, however the reason why the 12 laboratories did not participate is unknown. Still, we speculate that training may be required by some non-participating pertussis NRLs in the EU/EAA.

Limitations

The panel and control strains were not tested by all possible methods used in the EQA prior to shipment (e.g. we did not perform WGS).

There were issues with some shipments of DNA to participating laboratories resulting in short delays and re-shipment, which may have affected the DNA quality included in the panels.

Not all participating laboratories had received training for genotypic AST of *B. pertussis* prior to participating in this EQA.

There was a reduced number of participants compared to previous pertussis EQAs, which may have been due to time constraints in microbiology laboratories during the COVID-19 pandemic.

Of the 16 participating laboratories, three were not able to perform testing of the EQA panel samples.

This EQA round lacked phenotyping (MIC) AST components, which would have an added value and would more accurately reflect laboratory capacity.

6. Recommendations

Pertussis NRLs should be able to perform AST of *B. pertussis* isolates using DNA based approaches, especially when culturing is less and less performed among clinical/diagnostic laboratories. Pertussis NRLs should also be able to correctly interpret the AST results of *B. pertussis* isolates.

Due to the increase of macrolide resistant *B. pertussis* isolates in China, and some spread of macrolide resistant isolates to neighbouring countries, NRLs should be able to identify possible macrolide resistant *B. pertussis* isolates by direct typing with PCR, Sanger sequencing or WGS.

DNA-based AST should be done simultaneously with IS481 PCR on clinical samples. AST results can be considered reliable only if there is a positive IS481 finding showing presence of *B. pertussis*.

As access to WGS increases agreed criteria for analysis of isolates, theinterpretation of results are required and should be developed.

Hospital laboratories in EU/EEA countries should be encouraged to include *B. pertussis* DNA-based AST, but the importance of continuing to culture *B. pertussis* should also be clearly communciated and culturing should be encouraged.

Future work is required by ECDC/NRLs to prepare guidance for DNA-based AST identification.

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Annex 1. List of EQAs performed under the ERLNPert-Net External quality assessment scheme for *Bordetella pertussis*

Year conducted	Topic	Number of participating laboratories	Reference
2022	Antimicrobial susceptibility testing	16	European Centre for Disease Prevention and Control. External quality assessment scheme for *Bordetella pertus*sis antimicrobial susceptibility testing, 2022. Stockholm: ECDC; 2023.
2021	Vaccine antigen expression	12	European Centre for Disease Prevention and Control. External quality assessment scheme for *Bordetella pertussis* vaccine antigen expression, 2021. Stockholm: ECDC; 2023.
2020	Serology	17	European Centre for Disease Prevention and Control. External quality assessment scheme for *Bordetella pertussis* serology – 2020 data. Stockholm: ECDC; 2022.
2018	PCR	28	European Centre for Disease Prevention and Control. External quality assessment for the detection of *Bordetella pertussis* by PCR, 2018 – On behalf of EUPert-LabNet network. Stockholm: ECDC; 2019.
2016	Serology	25	European Centre for Disease Prevention and Control. External quality assessment scheme for *Bordetella pertussis* serology 2016. Stockholm: ECDC; 2018.
2013	Serology	21	European Centre for Disease Prevention and Control. External quality assessment scheme for *Bordetella pertussis* serology 2013. Stockholm: ECDC; 2014.
2013	*Bordetella* identification and *B. pertussis* typing	16	European Centre for Disease Prevention and Control. EQA scheme for *Bordetella* identification and *B. pertussis* typing. Stockholm: ECDC; 2014.
2012	PCR	21	European Centre for Disease Prevention and Control. External quality assessment scheme on PCR for *Bordetella pertussis*, 2012. On behalf of the EUPert-LabNet network Stockholm: ECDC; 2012.

Annex 2. List of participating laboratories

Country	Laboratory/institution
Austria	Austrian Agency for Health and Food Safety GmbH (AGES)
Belgium	Universitair Ziekenhuis Brussel
Croatia	Croatian Institute of Public Health
Czech Rep	National Institute of Public Health
Denmark	Statens Serum Institut
Estonia	Laboratory of Communicable Diseases
Finland	University of Turku
France	Institut Pasteur
Germany (Graz,Austria)*	D&R Institute for Hygiene, Microbiology and Environmental Medicine
Hungary	Legionella-Bordetella Lab
Iceland	Landspitali University Hospital
Ireland	Molecular Microbiology – Children's Health Ireland at Crumlin
Italy	Istituto Superiore di Sanità - Rome
Romania	Bacterial Respiratory Infections Laboratory/ "Cantacuzino" National Institute for Medical Military Research and Development
Slovakia	Regional Authority of Public Health Banská Bystrica
Slovenia	National Laboratory of Health, Environment and Food

**The National Pertussis Reference Laboratory of Germany is located in Graz, Austria.*

Annex 3. The ERLNPert-Net EQA scheme for *Bordetella pertussis* antimicrobial susceptibility testing, 2022

Specific contract ECDC/2019/023 work package 2 - instructions and reply form

11 April 2022

Coordination of the European Reference Laboratory Network for Pertussis (ERLNPert-Net), Contract reference: OJ/2019/OCS/10548 and regarding the Framework Contract (FWC), ECDC/2019/023.

Work package 2: EQA scheme for identification of macrolide resistant/sensitive *Bordetella pertussis* isolates among the National Reference Laboratories in Member States, Iceland, Liechtenstein and Norway.

Background:
Macrolides, such as azithromycin and erythromycin, are first-line drugs for the (prophylactic) treatment of pertussis. A point mutation of A2047G in the 23S rRNA gene is associated with *Bordetella pertussis* resistant to macrolides.

EQA panel:
11 x *Bordetella pertussis* DNA in water tubes
EQA01, EQA02, EQA03, EQA04, EQA05, EQA06, EQA07, EQA08, EQA09, EQA10 and EQA11

2 x *Bordetella pertussis* DNA from control strains
S-control: DNA from *B. pertussis* isolate sensitive to macrolides
R-control: DNA from *B. pertussis* isolate resistant to macrolides

Note: The *B. pertussis* DNA is stored and delivered in 1.5mL Eppendorf tubes (RNAase/DNAase free)

Sample dilutions
All samples are pre-diluted in ultrapure water and no further dilution should be done. Each tube contains 50µl of sample.

IF THE SHIPMENT HAS ARRIVED FROZEN
We would recommend storing all the tubes containing the *B. pertussis* DNA at -20°C (short term) or in -80°C (long term).

IF THE SHIPMENT HAS UNFORTUNATELY ARRIVED THAWED
DNA should be stable for a relatively long period in room temperature, even when diluted in water. However, we recommend to store them in -20ºC if the testing is not started immediately. If this would happen, please indicate this within the reporting phase as it may have an effect on the results.

Instructions
We encourage the participants to use their in-house methods or those methods available to detect macrolide resistance among the EQA panel samples. If PCR based approach is used, we recommend to use your own controls in addition to those included within the panel. In addition, if you chose to use qPCR or block-based PCR, we would like to recommend to perform three independent assays for result confirmation.

Study objectives:

1. To assess the ability of each laboratory to correctly report the sensitive and resistant *B. pertussis* DNA of each EQA panel sample.
2. To assess the use of different methods used for identification of sensitive/resistant *B. pertussis* in the participating laboratory.

1. *B. pertussis* isolates sensitive or resistant to macrolides
Identification of macrolide sensitive or resistant *B. pertussis* DNA of each panel sample by PCR, sequencing or in-house methods. Two protocols for PCR based identification (UTU) are provided.

Some published methods for your consideration:

i) Protocol from UTU: Block-based allele specific PCR for identification of macrolide resistance of Bordetella pertussis (attachment in email)

ii) Protocol from UTU: High Resolution Melting Analysis for identification of macrolide resistance of Bordetella pertussis (attachment in email)

iii) Bartkus JM, Juni BA, Ehresmann K, Miller CA, Sanden GN, Cassiday PK, et al. Identification of a mutation associated with erythromycin resistance in Bordetella pertussis: implications for surveillance of antimicrobial resistance. J Clin Microbiol. 2003;41(3):1167-72.

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v) Guillot S, Descours G, Gillet Y, Etienne J, Floret D, Guiso N. Macrolide-resistant Bordetella pertussis infection in newborn girl, France. Emerg Infect Dis. 2012;18(6):966-8.

Note: you can use WGS for identification of macrolide sensitive or resistant *B. pertussis* DNA of each panel sample, although the protocol is not included.

Please enter your details and results below and return to UTU before Wednesday 3rd of August, 2022.

	***B. pertussis* isolates sensitive or resistant to macrolides**	
Name:		
Laboratory:		
Country:		
Method:	Block-based PCR	Y/N
	qPCR	Y/N
	Sequencing	Y/N
	Other	Please specify:
Primers used:		
Samples	**Final result: Sensitive or resistant *B. pertussis* DNA (S/R)**	**Final result: No DNA identified**
EQA1		
EQA2		
EQA3		
EQA4		
EQA5		
EQA6		
EQA7		
EQA8		
EQA9		
EQA10		
EQA11		
Control DNAs		
S-control, DNA from sensitive *B. pertussis*		
R-control, DNA from resistant *B. pertussis*		

Please enter final result for each panel sample in shaded column (S indicates sensitive, R: resistant, N: no DNA or equivocal)

Annex 4. Provided protocols for the participating laboratories to perform AST testing (optional)

UNIVERSITY OF TURKU

Block-based allele specific PCR **for identification of macrolide resistance of *Bordetella pertussis***

Background

Macrolides, such as azithromycin and erythromycin, are first-line drugs for the (prophylactic) treatment of pertussis. A point mutation of A2047G in the 23S rRNA gene is associated with *Bordetella pertussis resistant to erythromycin and azithromycin* [1]. An allele-specific PCR-assay has been developed to detect this mutation [2, 3].

Reagents

AmpliTaq Gold™ DNA Polymerase with Buffer II and $MgCl_2$, 250 U (Applied Bioystems™, cat N8080241)
dNTP Solution (Promega, U1240)
Dimethyl sulfoxide (Merck KGaA, cat 1.09678.0100)
Molecular biology grade water (Type I)
Agarose (SeaKem, cat 50005)
Midori Green (Nippon Genetics, cat MG04)
TBE Electrophoresis Buffer 10× (Thermo Scientific, cat B52)
Plus DNA Ladder 100 bp (Thermo Scientific, cat SM0323)
DNA Gel Loading Dye 6× (Thermo Scientific, cat R0611)

Primers:

Primer name		Sequence
FP_23S_rRNA	(forward)	GTGATGGGGTGCAAGCTCTT
RP_23S_rRNA	(reverse)	TCTGGCGACTCGAGTTCTGC
WP_23S-rRNA	(sensitive)	ATCTACCCGCGGCTAGACAGA

Preparing the DNA-template

Prepare the DNA template by taking couple of draws of *B. pertussis* culture from petri dish using the 10 µl loop. Suspend the bacteria in 300 µl molecular biology grade water (Type I) in an Eppendorf tube. Heat the suspension at 95 °C for 30 minutes. Centrifuge at 13 000 rpm for 5 minutes, and transfer the supernatant (liquid) to a new Eppendorf tube. Alternatively, extracted *B. pertussis* DNA can be used instead of water suspension

If the gel bands are faint, increasing the *B. pertussis* DNA concentration at the heated water tubes, or using an extracted *B. pertussis* DNA may improve the results.

PCR-reaction

Prepare the PCR reaction mixture in an Eppendorf tube according to Table 5. Include control strains ATCC 9797 (no mutation, sensitive) and NAP-12-30 (mutation, resistant) and also a negative control (sample without DNA) in every assay.

Table 5. PCR-reaction-mixture. *Total reaction volume is 50 µl.*

Reagents	One sample (µl)	Final conc.
Molecular biology grade H_2O	23	
10x buffer	5	1x
$MgCl_2$ (25 mM)	3	1,5 mM
dNTP's (2 mM each)	5	200 uM each
FP_23S_rRNA (10 µM)	2	20 pmol
WP_23S_rRNA (10 µM)	2	20 pmol
RP_23S_rRNA (10 µM)	2	20 pmol
AmpliTaq Gold (5 U/µl)	0,5	2,5 U
DMSO	2,5	5 %
Total	45	

Transfer 45 µl of the reaction mixture to a 0,2 ml PCR-tube and add 5 µl of DNA-template.

Run the PCR-amplification with the following program (Bio-RAD 1 program: 23s_RRNA):

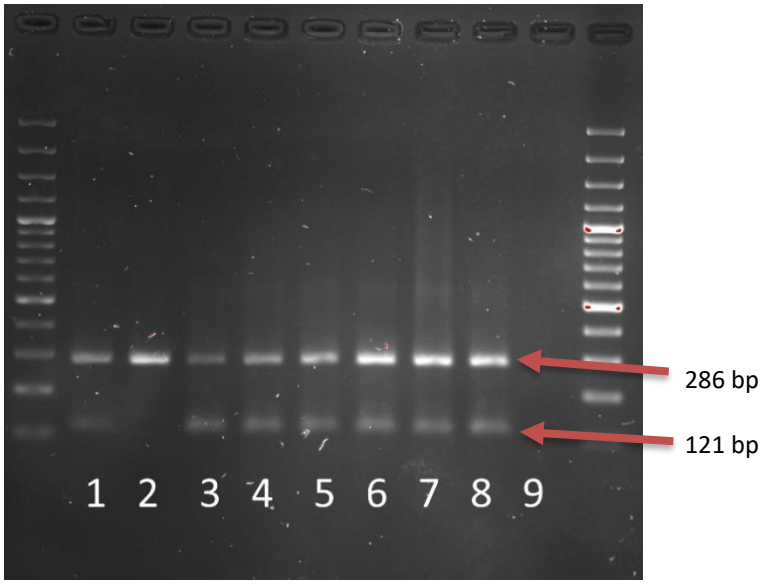
95 °C	15 min	
95 °C	1 min	x 40
58 °C	30 s	
72 °C	30 s	
72 °C	10 min	
10 °C	∞	

Gel electrophoresis and gel imaging

Use a 1,5 % agarose gel, stained with Midori Green.

Mix 5 µl PCR product and 2 µl loading dye and pipet them on the gel. Use 100 bp ladder for product size determination. Run the gel with 120 V for 60 minutes and use 1× TBE-buffer. Detect the bands with GelDoc XR+ Imaging System (BioRad, USA). Strains without the mutation will give two bands of 286 bp and 121 bp (Figure 1, lane 1, no mutation, sensitive). Strains with the A2047G mutation give only one band of 286 bp (Figure 1, lane 2, mutation, resistant).

Figure 1. Picture of the gel electrophoresis.



Lane (1) a control with no mutation, lane (2) a control with mutation, lanes (3-8) samples without the A2047G mutation and lane (9) a sample without DNA (water control). The ladders are 100 bp.

References:

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3. Wang Z, Han R, Liu Y, Du Q, Liu J, Ma C, et al. Direct Detection of Erythromycin-Resistant *Bordetella pertussis* in Clinical Specimens by PCR. J Clin Microbiol. 2015;53(11):3418-22.

Instruction for the PCR mix. Pipette: mix 45 µl and template 5 µl.

		Sample mix (µl)
Reagent	**1 á (µ)**	**8**
Molecular Biology grade H_2O	23	184
10x buffer	5	40
MgCl2 (25 mM)	3	24
dNTP's (2,0 mM each)	5	40
FP_23S_rRNA (10 mM)	2	16
WP_23S_rRNA (10 mMl) (sensitive)	2	16
RP_23S_rRNA (10 mMl)	2	16
AmpliTaq Gold (5U/ul)	0.5	4
DMSO	2.5	20
tot. V	45	360

Sample list		13	
1	ATCC	14	
2	NAP	15	
3	846	16	
4	847	17	
5	848	18	
6	849	19	
7	NTC	20	
8		21	
9		22	
10		23	
11		24	
12		25	

Gel run template (Ladder 100 bp, 120 V and 60 min):

Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sample	Ladder	ATCC	NAP	846	847	848	849	NTC	Ladder											

Controls:

ATCC 9797	NAP-12-30
Sensitive, no mutation (-)	Resistant, mutation (+)

High Resolution Melting Analysis for identification of macrolide resistance of *Bordetella pertussis*

Background

Macrolides, such as azithromycin and erythromycin, are first-line drugs for the (prophylactic) treatment of pertussis. A point mutation of A2047G in the 23S rRNA gene is associated with *Bordetella Pertussis resistant to erythromycin and azithromycin*. Since the nucleotide substitution from A to G results in a change in melting temperature of specific PCR product, with High-Resolution Melting Analysis (HRMA) this point mutation can be detected. The difference in melting temperature between the wild-type and the heterozygote variant is approximately 0.3 °C, which is shown in Figure 2 [1, 2].

Reagents

LightCycler 480 High Resolution Melting Master kit (Roche, Cat. No. 04 909 631 001)

96-well PCR plates (FrameStar® 96 Well Semi-Skirted PCR Plate, Roche Stylus, Plus qPCR Seal)

Molecular biology grade water (Type I)

Primers and product size:

Forward CCTGCACGAATGGCGTAA (HRM_A2047G_F1)
Reverse CCTCCCACCTATCCTACAC (HRM_A2047G_R2)
Product size 168bp

Controls:

ATCC 9797 (no mutation, sensitive)
NAP-12-30 (mutation, resistant)
Water tube (No DNA control)

Preparing the DNA-template (heated water - *B. pertussis* suspension or extracted *B. pertussis* DNA)

Prepare the DNA template by taking couple of draws of *B. pertussis* culture from petri dish using the 10 µl loop. Suspend the bacteria in 300 µl molecular biology grade water (Type I) in an Eppendorf tube. Heat the suspension at 95 °C for 30 minutes. Centrifuge at 13 000 rpm for 5 minutes, and transfer the supernatant (liquid) to a new Eppendorf tube and discard rest of the tube. Dilute the supernatant with biology grade water 1:1.

You can also use commercial kit for extraction of *B. pertussis* DNA to be used in the assay. Concentration of 1ng/ul of extracted DNA is recommended to be used.

PCR-reaction:

Total volume of the PCR-reaction is 20 µl: master mix 18 µl and DNA-template 2 µl. Prepare the master mix according to the table 1. Suspend, Do not vortex, the Master Mix gently with pipet and keep the HRM Master Mix cold as it contains the PCR-enzyme. When adding the template pipette on the well bottom and suspend it to the master mix.

Table 6. PCR master mix

Reagent	1 á (µl)	Final concentration
PCR grade water	5,4	
$MgCl_2$ (25 mM)	1,6	2 mM
Primer Fwd: HRM_A2047G_F1 (10 µM)	0,5	0,25 mM
Primer Rew: HRM_A2047G_R2 (10 µM)	0,5	0,25 mM
High Resolution Melting Master Mix (2x conc.)	10	
V(tot)	18	

Protocol for LightCycler run:

Program Name	Pre-incubation		
Cycles	1	Analysis Mode	None

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:10:00	4.40		0	0	0

Program Name	Amplification		
Cycles	33	Analysis Mode	Quantification

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:10	4.40		0	0	0
60	None	00:00:15	2.20		0	0	0
72	Single	00:00:10	4.40		0	0	0

Program Name	Melting curve		
Cycles	1	Analysis Mode	Melting Curves

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:01:00	4.40		0	0	0
40	None	00:01:00	2.20		0	0	0
65	None	00:00:01	4.40		0	0	0
95	Continuous		0.06	10	0	0	0

Program Name	Cooling		
Cycles	1	Analysis Mode	None

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
40	None	00:00:10	1.50		0	0	0

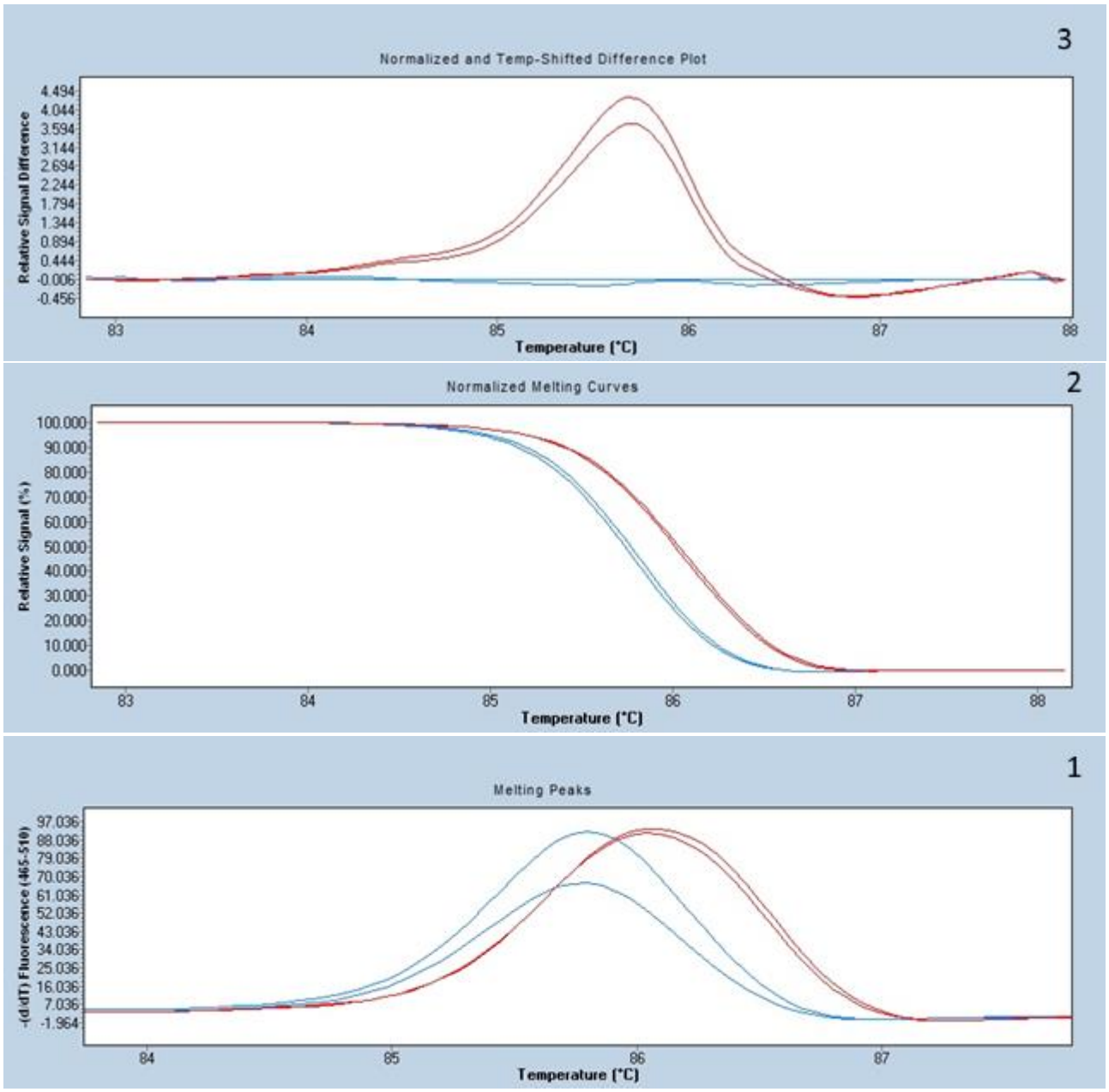
Analysing the results

For analysing the results, Gene Scanning from Analysis menu is used. Press the Calculate and the program groups the samples automatically according to controls. Sometimes the program auto groups the samples wrongly, and therefore it is advisable to check the melting peaks, the normalized melting curves and the temp-shifted difference plot (figure 1).

If the samples are not grouped, you can try following procedures to enhance it:

1. Change the setting Standards in run to Auto grouping.
2. Change the sensitivity from 0,30 to 0,45.
3. Adjust the slide bars on the normalization window.

Figure 2. The example pictures of melting peaks (1), normalized melting curve (2) and temp-shifted difference plot (3) of resistant (red lines, mutation) and sensitive (blue lines, no mutation) controls



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2. Zhang Q, Li M, Wang L, Xin T, He Q. High-resolution melting analysis for the detection of two erythromycin-resistant *Bordetella pertussis* strains carried by healthy school children in China. Clin Microbiol Infect. 2013;19(6):E260-2.

Appendix I. Pipetting scheme for PCR and sample plating

Instruction for PCR mix:

		MIX
Reagent	1 á (µl)	14
PCR grade water	5.4	75.6
$MgCl_2$ (25 mM)	1.6	22.4
Primer Fwd (10 µM)	0.5	7
Primer Rew (10 µM)	0.5	7
High Resolution Melting Master Mix (2x conc.)	10	140
V(tot)	18	252

Pipette: PCR mix 18 µl, template 2 µl.

Sample list:

	1	2	3	4	5	6
A	Pos9_DNA_10	Pos9_DNA_5	Pos_DNA_2.5	Pos9_DNA_1.25		
B	NAP_DNA_10	NAP_DNA_5	NAP_DNA_2.5	NAP_DNA_1.25		
C	ATCC_DNA_10	ATCC_DNA_5	ATCC_DNA_2.5	ATCC_DNA_1.25		
D	NTC					
E						
F						
G						
H						

Controls:

ATCC 9797	NAP-12-30
No mutation, sensitive (-)	Mutation, resistant (+)

Appendix II. Optimization scheme for MgCl2 concentration

$MgCl_2$ concentrations for optimizing the Mg^{2+}.

Concentration (mM) / 20 µl	1	1,5	2	2,5	3	3,5
$MgCl_2$ (25mM) (µl)	0,8	1,2	1,6	2	2,4	2,8
PCR water (µl)	2,2	1,8	1,4	1	0,6	0,2

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