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Outbreak investigation: how to prevent laboratory contamination during high throughput testing

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N. Renevey & B. Thür*

Diagnostics Department, Institute of Virology and Immunology, Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland

*Corresponding author: barbara.thuer@ag.ch

Summary

Early disease identification is essential for the rapid implementation of (bio-)security measures and disease eradication. Thus, both speed and accuracy of diagnosis are required should an outbreak occur. This is particularly important for outbreaks of highly contagious animal diseases. Efficient and correct testing as well as the reduction of technical breakdowns should be addressed by a laboratory contingency plan specially designed for the use of polymerase chain reactions (PCRs), i.e. PCR or reverse transcription PCR ([RT]–PCR) or the high throughput quantitative real-time form of these tests ([RT]–qPCR). Problems with contamination during outbreak investigations can negatively impact reliable diagnostics, and ultimately the eradication of the disease in the field. Therefore, well-defined standard procedures for preventing contamination are highly recommended, particularly in emergencies when a steep increase in the number of samples necessitates the use of additional, less experienced laboratory support staff. In the context of an outbreak, the possible implementation of preclinical testing is an important point of discussion. Very sensitive methods, which are prone to contamination, are used for this type of testing, increasing the risk of false-positive results. Potential approaches to minimising contamination include standard protocols adapted for high throughput analyses, different

procedures for samples with an expected high or low viral load, and the prioritisation and treatment of samples with respect to epidemiological aspects (e.g. epidemic vs endemic situations). Although there may be pressure to perform outbreak investigations as soon as possible, the authors do not recommend preclinical testing in every case due to the severe consequences of false-positive results. Contingency plans should be discussed with the responsible authorities and communicated to relevant personnel before an outbreak occurs.

The specific aim of this paper is the detailed description of solutions to prevent the laboratory contamination of samples and to avoid the generation of false-positive results.

Keywords

High throughput testing – Highly contagious animal disease – Laboratory contingency plan – Outbreak – Prevention of contamination – Real-time reverse-transcription polymerase chain reaction – RT–qPCR.

Introduction

Many countries are free from highly contagious diseases but as globalised trade and international traffic are increasing, the risk of the accidental introduction of animal diseases, such as foot and mouth disease (FMD), classical swine fever (CSF) or African swine fever (ASF), is rising. Therefore, it is important for countries to be prepared for such emergencies. This is the main reason why Member States of the European Union (EU) develop emergency plans in which the necessary national measures are defined to ensure a high level of disease preparedness and awareness. EU legislation requires that Member States have laboratory contingency plans (e.g. Council Directive 2001/89/EC on CSF [1] and Council Directive 2003/85/EC on FMD [2]). For CSF diagnostics, it is recommended that provision is made within plans for appropriate resources, including laboratory staff, equipment and infrastructure, to deal with an emergency (3, 4). Laboratory contingency plans have also been discussed

internationally, and different countries have set down national laboratory contingency plans (3, 4). The World Organisation for Animal Health (OIE) has published a list of countries which have national contingency plans for animal disease outbreaks (5). Therein, most of the countries also mention laboratory contingency plans. In some countries, for example Australia, contamination and decontamination are topics of emergency preparedness but its contingency plans mainly focus on preventing the spread of infectious agents between laboratories and the field (5). Nevertheless, to the authors' best knowledge, no laboratory contingency plan is currently published which places emphasis on the prevention of sample contamination in the laboratory. The authors assume, however, that a number of laboratories have implemented their own unpublished prevention measures. Also, in the diagnosis of human diseases, a lack of test and sample standardisation, as well as false-positive results caused by contamination, are relevant topics in times of crisis (6).

It is important to know in advance how samples are going to be handled in emergencies, with the decision already made as to which samples should be obtained during the outbreak, which diagnostic tests will be performed and in which areas of the laboratory the different steps will take place.

During the past few decades, either polymerase chain reaction (PCR) or reverse transcription polymerase chain reaction (abbreviated together as [RT]—PCR) or the quantitative form of these tests ([RT]-qPCR) have become important tools in the diagnosis of highly contagious diseases like FMD, CSF, ASF or avian influenza (AI) (2, 7, 8, 9, 10, 11, 12). As these methods are very sensitive, rapid and suitable for high throughput testing of samples, the swift identification of diseases is possible, an action that is crucial for the rapid implementation of security measures and successful disease eradication. In this context, preclinical testing represents an additional important point within outbreak plan preparation, which has recently gained more attention (13, 14, 15, 16). However, the very sensitive testing methods that are required for this kind of examination, increase the risk of false-positive results due to contamination (10).

When there are no outbreaks, false-positive results can be recognised and handled using test repetition or confirmatory tests, and the contamination source can be investigated. In emergency situations, however, with a large increase in the number of samples to be tested, time-consuming additional testing is often not possible.

EU Member States should ensure that, in accordance with their approved contingency plans, real-time outbreak simulation exercises are performed (2). Switzerland, as an EU-associated country, carried out a one such simulation exercise in 2011. The large scale national disease drill, called NOSOS (Greek expression for 'disease'), was organised by the Swiss Federal Food Safety and Veterinary Office (FSVO) and was on FMD (17). This simulation exercise involved farmers, field veterinarians as well as the Institute of Virology and Immunology (IVI), which is the national reference laboratory for FMD in Switzerland. Unfortunately, on day three of the exercise, the diagnostics laboratory was confronted with a contamination in RT–qPCR, leading to false-positive results. When the contamination was recognised, there was no time to search for its origin (17). As a result, many samples eventually had to be retested and the RT–qPCR method had to be replaced with a method detecting another gene sequence. This led to considerable delays as the possibility of contamination in the laboratory had not been addressed in the contingency plan. NOSOS demonstrated the importance of preventative steps in avoiding contamination during high throughput testing.

In this context, the contamination of samples can be defined as the unintended addition to samples of the infectious target, target genome or artificial genome sequences, especially those used in research (such as plasmids). Contamination through infectious virus or nucleic acid carry-over can occur at all stages of sample processing from sample collection to sample testing; therefore, steps should be taken to prevent cross-contamination along each step of the process.

In this paper, the authors address some aspects of laboratory preparedness which will help to avoid the problems inherent in sample contamination, and possible solutions to such problems, mainly in an

outbreak situation. The authors thereby assume that the normal standard of good laboratory practice (GLP) will already be in place in the laboratory in which these preparedness plans will be implemented; thus, the subject of GLP has not been covered in this paper.

Impact of incorrect diagnostic results during outbreak investigations and relevant questions and decisions

The impact of false-positive or false-negative laboratory results when testing for highly contagious diseases is different in former disease-free countries or regions when compared to endemic countries; for example, it dictates whether restriction measures are put in place or not. In the case of false-positive results in disease-free countries, the possibility exists that herds would be killed and/or trade and movement restrictions would be implemented in a region or even a whole country for no valid reason. In contrast, missed cases derived from false-negative results can lead to the rapid spread of the disease (18, 19).

In the context of emergency preparation for an outbreak, the following questions have to be discussed and addressed. It is important to note that for some of these countries, it is essential to discuss the questions with the responsible authorities and emergency control teams before an outbreak occurs in order to determine the level of and procedure for testing necessary:

– Is it important that the test has high sensitivity or high specificity?

– Is it necessary to have very sensitive methods, despite the fact that they are potentially more prone to contamination?

– Is the diagnosis going to be made at the herd level or at the individual animal level?

– Is it desirable to have a perfect test system with confirmed results that rely on extended investigations and intense confirmatory testing, bearing in mind that this requires significant personnel resources and could therefore raise problems of laboratory capacity?

– In a situation of insufficient laboratory capacity, which samples should be given priority and how should this be determined?

– Should eradication rely more on clinical investigation, which is perhaps less sensitive and specific than laboratory investigation, or on on-site testing on farms?

– Is early detection using preclinical testing and highly sensitive methods needed?

The answers could be diverse for different countries or laboratories, depending on their disease statuses, laboratory capacities, financial resources, methods used, available equipment, etc. In a former disease-free region, it is perhaps preferable to have an almost perfect test system with high specificity and high sensitivity, which needs confirmatory testing. During an outbreak, when costly measurements such as trade restrictions are already in place, it might be preferable to accept false-positive rather than false-negative results. Although false-positive results can lead to the culling of healthy herds and to an increase in epidemiological investigations, such as tracing back and forward, false-positive cases might be preferable to undiagnosed false-negative ones. The strategies based on the answers to the questions listed above that were formulated with the competent authorities should then be transparently communicated to stakeholders so they are able to understand and apply this strategy.

Weight of false-positive results due to contamination, in the context of an outbreak

False-positive results can be markedly reduced by confirmatory testing, which is normally undertaken during the diagnosis of the first case in an outbreak (also called the index case).

Although false-positive results originating from a contamination event should always be followed up on, their impact could be more substantial if:

– there is no time for confirmatory testing, such as in emergency situations when there is a large increase in the number of samples being tested

– the laboratory receives a large number of strong-positive samples

– highly sensitive tests are needed due to small amounts of analyte in the majority of samples

– samples from different populations, which may be negative, weak positive and strong positive, have to be tested in parallel.

A possible contamination with a crucial impact is disease spillover between different epidemiological units (herds). During outbreak investigations, the differentiation between contaminations within an epidemiological unit or between two different epidemiological units is important, since it impacts the eradication of the disease.

It is the opinion of the authors that in the diagnosis of viral animal diseases, contamination can occur; through three sources: virus particles, nucleic acid and antibodies. The authors focus in this paper is on contamination prevention for laboratory methods which have a higher likelihood of contamination and on the most relevant methods used during outbreak investigations (see Table I). Virus isolation through cell culture or embryonated eggs is a sensitive reference detection method (highlighted in orange in Table I) for many OIE listed viral diseases (9, 10, 11, 20), and it generates a high amount of virus particles. However, virus isolation is, in general, less sensitive than nucleic acid amplification assays, which are highly sensitive and generate a very high amount of target genome during amplification (highlighted in red in Table I). Regarding virus isolation, only infectious virus will result in contamination, but for nucleic acid amplification assays, contamination can occur with fragments of viral genome. Fischer *et al*. have shown that it is difficult to remove fragments of viral genome from the environment (21). Therefore, the most susceptible methods for contamination that are in general use in outbreak diagnostics are molecular-biology-based methods (22, 23). In addition, the authors assume that in outbreak testing, nucleic acid

amplification methods are more frequently used (highlighted in red in Table I) than virus isolation. The latter's less-frequent use during an outbreak (highlighted in green in Table I) is due to the fact that it is less suitable for fast and high throughput diagnostics (10). In addition, antibody detection with enzyme-linked immunosorbent assay (ELISA) is a widely used method, but, to the authors' knowledge it is not prone to contamination and does not represent a topic of concern in the field of diagnostics. Therefore, the focus of this paper is on preventing contamination in nucleic acid amplification methods, namely (RT)–qPCR. Since these methods are currently in frequent used during first-line testing (10, 11, 12) and as there is not always time for the confirmation of results in an outbreak situation, contamination prophylaxis is particularly important.

It is especially worth mentioning preclinical (RT)–qPCR testing. All points listed above could be true for preclinical testing, the use of which is increasingly considered in outbreak investigations for FMD, CSF and ASF. For example, preclinical testing was used in primary outbreak testing in the United Kingdom (UK) in 2007 (13, 14, 15, 16). Preclinical samples often consist of blood samples, as signs such as lesions and indeed dead animals will not yet be detectable. As these samples are obtained very soon after a possible infection, they frequently contain only a small amount of virus and the method of choice for analysing these samples is (RT)–qPCR (24, 25). Consequently, results will be negative or only weak positive and, therefore, it can be difficult to differentiate a true weak-positive reaction from contamination.

Besides sensitivity, another important requirement for the analytical methods is that results are generated quickly, without any delay, such as tests which take a long time to perform or re-testing and confirmatory testing, in order to find the positive herds as quickly as possible. In parallel with testing the mostly weak-positive preclinical samples, strong-positive samples from animals with clinical signs may also need to be investigated. Based on the experience of the authors and reports in the literature, this is the case, for instance, in vesicular fluid from cattle or pigs with FMD with high amounts of viruses (26,

27). In an ASF outbreak, it is possible that samples from dead or sick pigs, which contain up to 10^8 genome copies (28) or more than 10^6 to 10^8 50% haem-adsorbing doses (HAD_{50}) (28, 29) per millilitre of blood, have to be tested in parallel with preclinical samples from herds in contact with infected premises. Therefore, it is recommended that several strategies are put in place in order to minimise the risk of cross-contamination.

Major contamination problems in nucleic acid detection methods and measures to minimise them

Contamination events can occur at several stages of sample processing. At the beginning of the diagnostic process, the carry-over of virus particles is the main source of potential contamination; for example, if samples are sent to a laboratory in tubes that are externally contaminated with a virus or if during centrifugation, tubes containing strong-positive samples contaminate others through virus-containing aerosols. At later stages of the diagnostic process, the problem with nucleic acid carry-over could become more relevant; for example, if samples or solutions are handled in laboratory rooms containing amplified genome from a previously performed analysis of positive samples.

The authors analysed diagnostic processes with reference to critical points for contamination, and proposed several possible measures to prevent this problem. Potential strategies and solutions to limit the risk of contamination, summarised in Table II, are discussed below.

General recommendations according to good laboratory practice

General topics concerning GLP (23, 30) will not be discussed in detail here. The most important general measure to avoid contamination is to have a strict and clear concept of which rooms will be used for which purpose. Keeping in mind that (RT)–qPCR should be performed directionally along the processing steps, whenever possible, different rooms or separated areas, containing suitable cabinets and sets of

equipment, should be used for each different step. Most importantly, rooms where amplification steps take place must be separated from other areas in the laboratory. All rooms should be routinely cleaned with effective cleaning or decontaminating agents, and the use of a cleaning calendar should be standard. Room-specific coats should be worn and room-specific material (e.g. gloves, racks, pipettes) used. A colour code can be employed to indicate which items belong to which room. Other laboratory work, such as virus isolation from cell cultures, incubation of embryonated eggs or pen-side testing (lateral flow devices), dealing with high amounts of virus should be undertaken in biosafety cabinets (BSC) or, preferably, in different rooms. Basic research should also be performed in separate rooms, mainly if the same genome sequences of viruses are being worked upon as diagnosticians are looking for in field samples. Moreover, the same people should not use rooms for diagnostics and research without clear instructions and safety measurements. Training on and sensitisation to contamination issues (e.g. touching door handles, etc.) and adherence to behavioural rules should be the norm. Laboratory contingency planning should address the increased need for space and personnel in outbreak situations, anticipate the maintenance of a clear room concept and control the activity of people.

Specific measures to diminish the risk of contamination

Table II summarises a number of measures to diminish the risk of contamination. As the first steps that take place in the laboratory, the receipt and registration of samples are a potential source of sample contamination with viruses, mainly in outbreak situations where a large number of samples, both positive and negative, normally arrive. To avoid the distribution of virus within the laboratory, the authors propose to create a sample-receiving area, separate from the laboratory area, where testing is performed, incoming sample containers can be cleaned or even decontaminated, and the potentially contaminated examination request form can be electronically scanned (**solutions a and b**). Another measure could be to handle samples from one epidemiological unit separately to others before samples, such as blood tubes, are opened for the first time in order to reduce the

risk of cross-contamination between herds or locations, since cross-contaminations are usually much more relevant than contaminations within one epidemiological unit (**solution c**). For effective separation, it is necessary to change gloves or clean hands and to decontaminate benches, racks and the exterior of pipettes between processing materials from different herds (**solution d**). Solutions c and d can be challenging when a lot of herds with only a small number of samples need to be tested. This point must therefore be addressed in the contingency planning. Nevertheless, in preclinical testing, where often a lot of samples from the same herd have to be tested, these solutions could be a practicable option.

When samples are opened for the first time, preferably in a BSC with ultraviolet (UV) irradiation, it is recommended that several aliquots are prepared, on the proviso that the number of samples and time pressure allow for this procedure. One of these aliquots should be stored as a 'clean' reserve that could be used in case of a sample contamination and one or two other aliquots should be used for testing (**solution e**). The type of tube utilised for aliquoting is relevant and it is preferable to use tubes fitted with a safety lid and a rubber seal to avoid leaking. Furthermore, the tubes should be tested in any event before an outbreak to be sure that they do not cause any problems (**solution f**). Safety lids are also useful to prevent virus carry-over between samples of the same epidemiological unit.

The centrifugation of samples, before or after aliquoting, is normally necessary to prepare material for investigation. Placing only those samples from one farm into a specific centrifugation cup (**solution c**) and using closed cups (**solution f**) can help to diminish cross-contamination between herds. The same procedure should be followed for sample homogenisation, for which open and closed systems are available. Strong-positive material should be processed with a closed system, whereas an open system, such as a laboratory mixer, may be adequate for weak-positive sample material, but is not optimal. The authors tested the TissueLyser II from QIAGEN (Venlo, the Netherlands) for contamination susceptibility (**solution g**). As the tubes do not have a safety lid, that the likelihood that virus carry-over

between the tubes could occur could not be ruled out. In order to test this possibility, the authors filled half of the tubes of each of the two 24-sample adapter sets with water and the other half with strong-positive samples, and performed the homogenisation. This experiment was repeated twice. The water samples were extracted using Trizol® (Thermofisher, Waltham, Massachusetts, United States of America) and tested in an RT–qPCR for viral contamination. Since all water samples remained negative (quantification cycle value [Cq-value] = 50), it seems that the closed TissueLyser system using tubes without safety lids can be considered safe (N. Renevey and B. Thür, unpublished data, 2013).

Depending on the disease, it should also be discussed if there are very strong-positive samples to be tested during an outbreak. The testing of such samples along with negative samples could be a crucial point for contamination. Samples with high concentrations of virus contain a higher carry-over risk during laboratory testing than weak-positive samples. One part of a solution might be to handle strong-positive material separately (**solution h**). For that, it is important to be able to estimate the potential viral load of a sample. Epidemiological information, such as vaccination status, signs, age of lesions and number of diseased animals can be used to estimate the virus load. Such information can provide an indication as to the presence of virus in a sample. In addition, knowledge of the tissue tropism and replication characteristics of a specific virus can help in estimating the viral load in a sample. For example, in animals infected with FMD virus (FMDV), high virus load is mainly found in epithelial lesions and vesicular fluid during the acute stage of infection (26, 27). Whereas for many other viruses, such as AI virus (AIV), the preferred site of viral replication, viral titres as well as the way of virus excretion are influenced by the infected animal (species, age) and the particular virus strain (31, 32).

In order to estimate the virus load, rapid tests such as pen-side tests or lateral flow devices (LFDs) can be used (**solution i**); this is true for FMDV analyses (33), for instance, where strong-positive samples would be detected by this method. The availability of separate rooms

is crucial in order to perform such tests; otherwise, the risk of contamination is even more enhanced. Samples that tested positive should not be further processed unless confirmatory testing, such as sequencing, is absolutely needed. In this case, samples should be diluted for subsequent testing. As the method is not very sensitive, samples that test positive in LFDs must contain a considerable amount of virus (34, 35). An LFD can also be used for AIV analysis in swab and feather specimens. Belák *et al.* observed that samples with a Cq-value lower than 25 were LFD-positive and those that had a Cq-value between 25 and 36 were LFD-negative (34, 35). Also for ASF virus (ASFV) detection, LFD tests are in evaluation (36). As these tests often have a high specificity but limited sensitivity, negative results should be interpreted with caution (34, 37). Thus, there are useful tools when undertaking a first sorting of strong-positive samples from other samples, but this adds time to the testing process and requires extra resources.

A prudent reduction in sample testing is an additional possibility (**solution j**), as overloading laboratory capacity can lead to contamination and the selection of a more representative smaller group of samples can help to identify a contamination. Therefore, it can make sense to test only those samples with the highest chance of being virus positive. For example, it would not be necessary to investigate vesicular fluids and blood samples from the same FMD-suspected animals, as, in general, vesicular fluid will provide a stronger positive than blood and is therefore the sample of choice. Apart from well-considered sample selection, pooling can also substantially reduce the number of samples. Tests should be performed on which and how many samples should be pooled, preferably before an outbreak, and the subject should be discussed with competent authorities.

The risk of contamination in nucleic acid extraction is strongly influenced by the method used (**solution k**). In an open robotic system, pipette arms with leaking tips moving over tubes or sample plates represent a potential source of contamination. Robots therefore should be precisely validated for their intended use. The authors

evaluated the semi-automated extraction robot TECAN Freedom EVO (Tecan, Männedorf, Switzerland) and found contamination (B. Hoffmann, personal communication, 2005). Other extraction robots such as QIAcube (QIAGEN), KingFisher Flex (Thermofisher, Waltham, Massachusetts, United States of America) or MagMAX Express-96 (Applied Biosystems, Foster City, California, United States of America) seem to be contamination safe (38, 39). Nevertheless, the authors performed an experiment to assess the contamination risk of the QIAcube robot, which extracts 12 samples in one run. Different runs were performed as follows:

- one strong-positive (Cq-value = 13) sample and 11 water samples:
- six strong-positive samples and six water samples
- eleven strong-positive and one water sample.

The extracted ribonucleic acid (RNA) was analysed with RT–qPCR and all water samples gave negative results (Cq-value = 50). Under these conditions, the authors considered the QIAcube to be safe (N. Renevey and B. Thür, unpublished data 2013). Depending on the extraction system, it could be advantageous to use one machine to process potentially weak-positive or negative samples and another, preferably a closed system, to process potentially strong-positive samples in order to prevent cross-contamination. Another solution (**solution l**) would be to dilute strong-positive samples before RNA and DNA extraction, for example, samples that were LFD-positive or samples originating from cell culture inoculation and showing cytopathic effect. As described above, the dilution should ideally be performed in a separate room (see **solution i**). In the authors' experience, supernatant of FMDV-positive cell cultures showing cytopathic effects (CPEs) can be diluted a minimum of 10^6 times to obtain $<10^{0.5}$ 50% tissue culture infective dose $(TCID)_{50}$/ml. This dilution still resulted in an RT–qPCR positive (N. Renevey and B. Thür, unpublished data, 2013).

Nucleic acid contaminations are mostly a problem in two-step RT–PCRs where complementary deoxyribonucleic acid (cDNA) is transferred to a separate tube for the PCR step (**solution m**). The risk of contamination is increased since tubes have to be opened twice and therefore strict measures against contamination are necessary (23). This kind of carry-over contamination is minimised by using a one-step RT–PCR (30).

In order to have a reliable contamination control procedure, it is essential to run several non-template controls (NTCs) as well as extraction controls, in parallel with the diagnostic samples (40). The lower the amount of nucleic acid that is expected within a sample, the more controls must be included in a sample run. The use of more NTCs enhances the possibility of detecting weak contamination (**solution n**).

When running (RT)–qPCR, the plate has to be sealed either with optical caps or a validated high-quality plastic sheet. If the latter is used, the validation process must be clearly defined (**solution o**). The authors' own experience shows some plastic sheets do not block the openings to the reaction plates sufficiently, possibly leading to evaporation that could contaminate the thermocycler or other samples.

In weak-positive samples, where contamination during (RT)–qPCR is possible, retesting the extracted RNA/DNA in a separate (RT)–qPCR run where only weak-positive or negative samples are tested should be considered (**solution p**). Additionally, a second (RT)–qPCR that amplifies another genome region can be used (**solution q**). Such a reserve (RT)–qPCR must be properly validated in advance and have known sensitivity, preferably equal to or better than the first line test, to confirm weak-positive samples.

Uracil-N-glycosylase treatment (41, 42, 43), which hampers carry-over contamination through amplified DNA, can also be used in (RT)–qPCR (**solution r**). This method would mainly be relevant in an outbreak situation where a lot of positive samples need to be analysed. But it cannot solve carry-over contamination, if basic research is performed in the same laboratory.

An amplified genome could also be a risk when autoclaved with other waste from the laboratory, since the possibility that vapour or air from the autoclave contains amplified target genome cannot be ruled out. Therefore, waste from genome amplification should not be autoclaved close to the laboratory where diagnostics is performed (**solution s**).

Specific considerations if contamination is present

The most important steps include cleaning the work area and laboratory equipment with a potent decontamination solution and replacing the PCR reagents. Although manufacturers often promote their products as effective for RNA/DNA decontamination, studies performed in the authors' laboratory and at the Friedrich Loeffler Institute (FLI), Greifswald, Insel Riems, Germany (21) showed the inefficiency of several decontamination solutions in destroying amplified (RT)–qPCR products. These and other studies demonstrated that hypochlorite solution is very efficient in destroying short (RT)–qPCR amplicons (44).

In addition, the interpretation of results represents a crucial step. In order to be able to differentiate between a positive result and a contamination, experience with the methods used and samples tested is required. For instance, material that normally has a high viral load, such as fresh epithelium and vesicular fluid in FMD, is expected to have low Cq-values, if sample quality is guaranteed. If vesicular fluid from a pig has a Cq-value of 39 only, the potential of contamination has always to be considered. The same is true, if a bovine blood sample has a Cq-value of, for example, 32 and epithelium from a tongue lesion from the same animal is negative. Thus, specialised knowledge of the investigated disease, including epidemiology, signs, pathology and suitable diagnostic material, facilitates, in many cases, the recognition of a potential contamination.

Contingency plan

In case of emergencies, a laboratory contingency plan is a significant advantage. It is essential to discuss the plan with competent authorities and those that have a role to play before its implementation in order to

ensure sufficient support for the strategy. Furthermore, it is important to know where and how increased numbers of samples are going to be handled, which supplementary devices and rooms could be used without increasing the risk of contamination and which additional trained personnel will be available for support. Some of the proposed solutions (e.g. **solutions c and d**, separation of samples) are difficult to implement in high throughput testing. Nevertheless, possible solutions should be discussed, and the advantages and disadvantages of the chosen procedure must be clear.

In general, molecular testing requires experienced laboratory workers familiar with contamination prevention. Thus, it is highly recommended that staff training is included in the contingency plan. When additional personnel are needed, which are not as well trained in methods and operational sequences as the existing staff, well-defined standard procedures for the prevention of contamination are highly recommended. Moreover, protocols that are used under normal circumstances need to be adapted to emergency situations, including the use of additional rooms, materials and personal staff, etc.

The authors also recommend developing an alternative testing strategy for emergency situations according to the needs which evolve during an outbreak. The requested sensitivity and specificity of the test system is an important feature of this strategy, and will be influenced by the reduction of the number of confirmatory tests in order to save time. The plan should consider measures to avoid and lower the impact of false-positive results due to contamination. For instance, knowing whether preclinical testing will be performed, how contamination can be prevented and what sensitive and quick alternative (RT)–qPCR protocols are available (possibly based on another genome localisation), are crucial. In contrast to clinical testing, in preclinical testing it is not possible to perform conventional PCR, virus isolation in cell culture or antigen ELISA as confirmatory tests.

In general, the sensitivity and specificity of the alternative tests that could be used in emergency situations in case of a contamination

event, must be known in advance in order for testing protocols to be changed immediately. It would also be helpful to determine in advance which confirmatory testing should be cancelled first when time and resources are in short supply, again with the agreement of all those who play a role. In addition, the laboratory should have a communication plan on how to inform stakeholders of a delay due to contamination or of a decrease in the validity of test results due to a reduction in confirmatory testing or the prioritisation of samples.

Discussion

In emergency situations where there is a significant increase in the number of samples to be tested, the level of time pressure, and the number of additional personnel required (who may not be well trained in methods and operational sequences), it could be challenging to follow the routine standard procedures to prevent contamination. Therefore, it is important to implement strategies in the laboratory contingency plan that will limit the chances of contamination. The contingency plan should address the testing regimen, which is intended to be used during an outbreak, but it should not be so complex that it compromises the workflow, which itself could be a contamination risk. In some situations, it would be better not to rely on the most sensitive method and/or not to test all samples, but to choose methods with a sensitivity previously agreed upon. It is also possible to test only selected samples rather than all, or to pool samples, again based on criteria previously agreed upon with all those who play a role. The sample selection is performed based on knowledge of virus distribution and tissue tropism, which allows samples to be chosen which have the highest chance of testing positive. In the context of fast and high throughput testing, and the limitation of confirmatory testing, false-positive results mainly in (RT)–PCR or (RT)–qPCR should be considered, as also mentioned in the EU diagnostic manual for CSF (45): 'the method is highly sensitive, but contamination may easily occur, which leads to false-positive results'. Also in microbiology laboratories dealing with human samples, contamination seems to be a major problem (22). In contrast with the gel-based (RT)–PCR, the reaction tubes of (RT)–

qPCR do not have to be opened after the reaction, which lowers the risk of release of amplified genome sequences and hence of contamination. Importantly, if laboratories also perform basic research in the same area as disease diagnostics, diagnosticians must act on the assumption that genomic material is present in the laboratory and must protect their samples by using separate solutions and equipment, for instance separate pipettes. It is also recommended that an area is dedicated specifically to diagnostics during an outbreak, and that the rooms and equipment that will be used are decontaminated once an outbreak is reported and before testing commences. Moreover, the correct interpretation of results, i.e. differentiating a contamination from a true positive, requires some experience. Precise knowledge of the test characteristics and validation data, and of the disease (including its epidemiology as well as the eradication strategy), may help the diagnostician in the critical interpretation of the results. Sufficient controls are also crucial in this regard.

Whether strong-positive samples should be separated from the remaining material for the prevention of contamination is less clear. Sample separation according to expected viral load will not always be easy to perform, especially if submission forms are not completed correctly, as is possible when field services are stressed. It is very important that the material sent into the laboratory is correctly and fully labelled. Therefore, submission forms should be easy to complete and the possibility that incorrect material is submitted should be minimised. Ideally, which material has to be sampled is indicated on the form. In addition, signs should be listed on the submission form. Since herd-based diagnosis is important for highly infectious diseases where control decisions will affect the whole herd, within-herd carry-over is generally not as critical as between-herd cross-contamination. Therefore, depending on the laboratory logistics, it may be important to separate samples from herds within the control zone from those outside this zone that are less likely to be infected. 'High-risk herd samples', rated according to epidemiological situation, could be processed together.

Experience in and knowledge of the analytical method used is needed to assess the susceptibility of a method to contamination, and some procedures have to be evaluated or validated before an outbreak occurs using strong-positive samples to show if they are susceptible to contamination. These validated methods should also be part of the contingency plan.

In the aftermath of the extremely challenging experience of an outbreak, personnel from those laboratories that were involved in sample testing should be relieved. However, for those countries that were free from a disease before an outbreak, the next phase is to regain their infection-free status. This often includes the serological testing of a larger section of the remaining animals or of the animals in repopulated holdings, which will be a new challenge for the laboratory (1, 2, 8). This topic was not the focus of the current publication; but also for this period, it is advantageous to have clear process descriptions already in place. It should, for example, be set out the following in a contingency plan:

- if the country would rely on emergency vaccination and which animals should be vaccinated;
- if a vaccination-to-kill or a vaccination-to-live strategy is planned;
- if it is possible to involve other laboratories in the serological testing and if such laboratories are already trained.

Although each outbreak is unique and not every situation can be anticipated, simulation exercises may provide some insight into potential improvements. Additionally, it is important to remember that the contingency plan needs to be flexible, since the situation differs between outbreaks (18).

In general, contingency plans could be an instrument with which to protect the laboratory from criticism and blame in case of false-positive or false-negative results during outbreaks. Also, for this reason, contingency plans should be supported broadly and should be communicated transparently.

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Table I
The most relevant methods in veterinary diagnostics, their susceptibility to contamination and importance during outbreak investigations

Method		Susceptibility to contamination	Importance of method during outbreak investigations
Isolation of infectious virus	Cell culture Embryonated eggs	• Very sensitive methods; one infectious virus can be enough to get a positive test result • Medium risk of contamination as high amount of virus can be generated during virus isolation	• Rarely used (as soon as the virus has been identified) • Mainly relevant for confirmation of the index case
Nucleic acid amplification	qPCR, RT-qPCR	- Highly sensitive methods, one single genome copy can be enough to get a positive test result - Generation of a very high amount of target genome during amplification - High risk of contamination if working with strong-positive samples or if basic research is done for the same virus in the same area	- Often used - High throughput capability - Fast test
	PCR, RT-PCR	- Sensitive methods - High risk of contamination because of the open system for visualisation of genome	- Rarely used - More laborious than qPCR or RT–qPCR - Sometimes used as a confirmatory test
Ag detection	Immunohistochemistry and LFD	- Not very sensitive methods	- Rarely used - If PCR is not available and strong-positive samples (sick animals) have to be tested
	ELISA	• Not very sensitive method • Low risk of contamination because a large amount of virus is needed to get a positive test result	• Rarely used • If strong-positive samples are expected (e.g. supernatant from cell culture with CPE, vesicular fluid in FMD) • For characterisation of virus type
Ab detection	ELISA	• Moderately sensitive method	• Often used and high throughput capability

HI VNT IIF	• Needs a considerable amount of Ab -positive sample for a contamination • Low risk of contamination when Ab are not yet present in samples during an outbreak	• Rarely used, mostly as a confirmatory test at the beginning of an outbreak

Contamination risk for outbreak investigations is determined as a combination of 'Susceptibility to contamination' and 'Importance of the method during outbreak investigations'

Ab: antibody
AG: antigen
CPE: cytopathic effect
ELISA: enzyme-linked immunosorbent assay
FMD: foot and mouth disease
HI: haemagglutination inhibition assay
IIF: indirect immunofluorescence
LFD: lateral flow device (pen-side test)
PCR: polymerase chain reaction
qPCR: quantitative real-time polymerase chain reaction
RT-PCR: reverse transcription–polymerase chain reaction
RT-qPCR: reverse transcription–quantitative real-time polymerase chain reaction
VNT: virus neutralisation test

Risk types:
Red/red: high risk
Red/orange, orange/orange or green/red: considerable risk
Green/orange: low risk
Green/green: very low risk

Table II

Measures to diminish the risk of contamination*

A	Scan potentially contaminated paper from examination requests
B	Decontaminate sample tubes or containers
C	Separate samples according to different farms to avoid contamination between epidemiological units
D	Change gloves and clean bench between different herds to avoid contamination between epidemiological units
E	Make several sample aliquots (retain samples)
F	Use closed centrifugation cups and tubes with safety lids and a rubber seal for centrifugation or homogenisation
G	Use a homogenisation system validated with regard to contamination risk
H	Separate samples according to material (viral load) based on knowledge of disease, epidemiology and virus distribution
I	Use a pen-side test (lateral flow devices) to categorise samples/farms according to their viral load
J	Reduce the number of samples to be tested based on knowledge of disease, epidemiology, virus distribution as well as by sample pooling
K	Use different extraction machines or methods validated with regard to contamination risk
L	Dilute strong-positive samples for extraction and (RT)–PCR/(RT)–qPCR
M	Avoid two step RT–PCR/RT–qPCR protocols
N	Use more NTCs with expected weak-positive or negative samples to recognise contamination
O	Use only validated high-quality plastic sheets for sealing (RT)–PCR/(RT)–qPCR plates otherwise use caps
P	Repeat weak-positive samples in a (RT)–qPCR run in the absence of strong-positive samples
Q	Use of a (RT)–qPCR that amplifies a part of another genome region
R	Use uracil-n-glycosylase with (RT)–PCR/(RT)–qPCR
S	Do not autoclave amplified nucleic acids

NTCs: non-template controls

PCR: polymerase chain reaction

qPCR: quantitative real-time polymerase chain reaction

RT–PCR: reverse transcription–polymerase chain reaction

RT–qPCR: reverse transcription–quantitative real-time polymerase chain reaction

*For detailed information on the measures, see the section entitled Specific measures to diminish the risk of contamination’