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Detection and differentiation of Schmallenberg, Akabane and Aino viruses by one-step multiplex reverse-transcriptase quantitative PCR assay

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The Schmallenberg virus (SBV), Akabane virus (AKAV), and Aino virus (AINV), a member of the Simbu serogroup within the genus *orthobunyavirus*, family *Bunyaviridae,* cause reproductive disorders including abortion, stillbirth and congenital malformation in ruminants. Because, the clinical signs are so similar, confirmatory diagnosis requires viral detection to differentiate infection between these three viruses. In this study, a one-step multiplex reverse-transcriptase quantitative PCR (one-step mRT-qPCR) was developed for the simultaneous detection and differentiation of SBV, AKAV and AINV. Oligonucleotide primers and probes were used to amplify the genes encoding the S segment of SBV (Accession No: HE649914), AKAV (Accession No: AF034942) and AINV (Accession No: AF034939). These conserved viral genome regions were chosen as the best candidates for the generation of specific primers and virus-specific probe sequences for SBV, AKAV and AINV. The detection limit for the one-step mRT-qPCR were 2.4 copies ($10^{0.6}$ $TCID_{50}$/ml) for SBV, 96.2 ($10^{1.5}$ $TCID_{50}$/ml) for AKAV and 52.3 copies ($10^{1.2}$ $TCID_{50}$/ml) for AINV. Various clinical samples such as bovine serum, bovine whole blood, bovine brain, goat serum and *Culicoides* samples were analyzed using the one-step mRT-qPCR and compared with previously published qPCRs. The field sample results were the same for the one-step mRT-qPCR and published qPCRs showing one bovine brain sample (1/123) being positive for AKAV. The one-step mRT-qPCR allows simultaneous detection and differentiation of three viral pathogens (SBV, AKAV, and AINV) that cause reproductive failure.

Molecular diagnosis of the Siberian sturgeon herpesvirus

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Aquaculture development inevitably entails increased fish viral disease problems due to the threat of extinction of valuable species of sturgeon and the increasing demand for sturgeon products in recent years. One highly dangerous and contagious disease of sturgeons is caused by Siberian sturgeon herpesvirus (SbSHV).

SbSHV is a DNA virus of genus Ictalurivirus Alloherpesviridae family Herpesvirales order, which belongs to a group of white sturgeon herpesvirus type 2 (Asipenserid Herpesvirus 2 - AciHV-2).

Siberian sturgeon herpesvirus disease causes great economic loses to Sturgeon production. The mortality of SbSHV disease is 100%.

Siberian sturgeon herpesvirus disease was first reported in Russia in 2006 during an outbreak in the Tver region. In the following years, the disease was reported in 7 regions of Russia, Kazakhstan and Finland.

Laboratory diagnosis based on virus isolation and identification by serological methods has some limitation such as a time-consuming.

The aim of this work is to develop a sensitive and specific real-time PCR assay that allows for rapid and accurate virus identification in the early stages of the disease.

Methods

Real time polymerase chain reaction (Real time PCR).

Results

Designed specific primers and an oligonucleotide probe complementary to the DNA polymerase gene`s part were the base of SbSHV of nucleotide sequence an analysis.

The evaluation of the developed test system specificity was performed by amplification of different SbSHV strains and isolates, heterologous viruses and normal cell and tissue cultures from healthy sturgeon.

We have examined samples received from the fish farms of Pskov, Vologda, Smolensk regions and from the Republic of Buryatia and Finland using the developed Real time PCR test system. Twenty-five of 49 samples tested were found positive.

Conclusion

The Real time PCR test system for the diagnosis of SbSHV has been developed. This test system allowing early identification of SbSHV genome is used for the monitoring of fish farms.

Development of a Real-Time PCR detection assay for identification and differentiation field and vaccine strains of myxoma virus.

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Myxoma virus (MYXV; family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Leporipoxvirus*) is a linear double stranded DNA virus that specifically infects rabbits and hares. Affected rabbits display conjunctivitis, anorexia, listlessness, and fever.

For specific prevention of myxomatosis live attenuated vaccines are used. Vaccinated animals in contact with field strains may become asymptomatic carriers of the virus and act as a reservoir of infection. In this connection, when an animal rabbit myxoma virus is detected, it is necessary to differentiate between vaccine and field strains.

The aim of this work is to develop a PCR detection method for the identification and differentiation of myxoma virus sequences, which allows for pathogen identification in pathological material and differentiating it from the vaccine strains and closely related viruses.

The primer and probe specificity was evaluated using a panel of DNA samples isolated from outbreaks of myxomatosis in Russia since 2009 till 2012, attenuated vaccine strains, DNAs of heterologous viruses, and also from DNAs isolated from organ samples of normal rabbits (including spleen, liver, and skin), and from the continuous cell line RK-13.

We analyzed nucleotide sequences of various MV strains available in Gene Bank database, and selected primers and a fluorescent TaqMan probe flanking MV Serp2 and M130R genes for MV DNA specific amplification.

Serp2 is a conserved gene of virulent and vaccine strains of myxoma virus, and thus differs significantly from other poxviruses. For discrimination of vaccine strains we selected a portion of gene M130R, which has a nucleotide substitution absent in virulent field strains.

RT-PCR analytical specificity levels were determined using DNA samples isolated from a series of tenfold dilutions of the virus material.

DNA isolation from the virus material was carried out using a modified nucleosorption assay with a sorbent (Boom et al, 1995). The RT-PCR was performed on a Rotor Gene-6000 cycler (QIAGEN, Germany).

The assay was performed using a standard reagent kit. The results were evaluated through the analyses of fluorescent signal accumulation curves for a FAM and Yellow channel using the software of the device used.

The studies found that the developed test can detect viral sequences in samples of various organs and tissues of naturally and experimentally infected animals. Moreover, the proposed kit could differentiate between of vaccine strains and field isolates of the myxoma virus.

The diagnostic specificity and sensitivity of the system was 100%. The calculated analytical sensitivity of the test was 1,5 lg ID_{50/sm^3}.

A New Level of Standardization in Rea-time PCR with the IDEXX RealPCR™ BVDV RNA Test

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Objectives:
Real-time PCR has revolutionized diagnostic testing over the past years. While real-time PCR is continuously developing, commercial assays often deliver a set of reagents designed for testing a precise number of samples for a specific target(s). IDEXX RealPCR real-time PCR tests aim to provide a new level of standardization to PCR diagnostics by using reagents in a modular system. The components of this modular system are shared over the entire test platform, making it possible to run any pathogen-specific detection mix with a standard master mix and a single positive control. Moreover, the IDEXX RealPCR modular system maintains a single cycling protocol for all tests and shared quality controls and guidelines across the entire platform.

Method:
The RealPCR BVDV RNA Test has been designed to detect Type I, Type II and HoBi BVDV. It has been evaluated using characterized samples and synthetic oligonucleotides. Different sample types have been tested including whole blood, serum, plasma and ear notches.

Results:
The test shows an analytical sensitivity of ≤ 15 copies / reaction for Type I, Type II and HoBi BVDV with efficiencies of > 95% over at least a 7-log range. The test displays no cross-reactivity with many common bovine viral pathogens either by in silico analysis or with diagnostic specificity testing. In addition to individual whole blood, serum, plasma and ear notch claims, the test detects BVDV in sample pools of up to 50 for blood fractions or 25 for ear notches.

Conclusions:
A rapid lysis protocol for ear notches has been validated, eliminating the need for full RNA extraction which greatly reduces sample handling and processing time. To ensure reliable results, the test employs a multiplexed internal sample control (ISC) to detect endogenous bovine RNA that i) eliminates the necessity of an internal positive control (IPC) spike and ii) controls for sample addition, sample integrity as well as proper RNA extraction and successful RT-PCR reaction.

Use of multiplex RT - PCR in real-time to detect Schmallenberg and Akabane viral sequences

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Schmallenberg and Akabane viruses – are arboviruses infecting cattle, sheep and goats, and causing serious economic losses due to drop in milk yield, a high level of abortions, stillbirths and mortality among newborns. The causal organisms belong to the family Bunyaviridae serogroup Simbu genus Ortobunyavirus.

Clinical signs of diseases caused by these viruses are similar. If an adult ewe or heifer is infected in the early stages of pregnancy, the fetal infection can occur, leading to abortion, birth of premature or dead fetuses and lambs, goats and calves with various malformations. Due to the similarity of the clinical symptoms, pathological changes and a high degree of genetic relatedness of viruses, developing methods for differential diagnosis is highly desirable. One methodological approach for differentiation of viruses is a multiplex polymerase chain reaction.

The aim of this work was to develop an assay to detect Schmallenberg and Akabane virus sequences using multiplex quantitative reverse transcription PCR (RT-qPCR) in real time.

«Primer Express» and «Oligo 6.0» programs were used to select appropriate primers. For detection of amplification products selected Taq-man probe technology, containing the 5'-ends of the emitters fluorescence FAM for Akabane virus, HEX for Schmallenberg virus, and the 3'ends - quenchers BHQ1 and BHQ2 respectively.

To evaluate the specificity of the test RNA from Schmallenberg, Nairobi sheep disease, Akabane, bluetongue and Rift Valley fever viruses was examined. RNA samples extracted from BHK-21/13 and CV-1 cells, blood samples and organs from Akabane virus – infected sheep and mice and from normal sheep.

Analytical sensitivity of the method was determined using in vitro transcripts synthesized on the template of the recombinant plasmid with the Schmallenberg and Akabane viruses genomes fragments insertion. Ten-fold serial dilutions of in vitro transcribed RNA (of known concentration) were used to determine analytical sensitivity of RT-qPCR.

The limit of sensitivity considered the maximum dilution at which a positive result was registered. The calculated value of analytical sensitivity of RT-PCR in real-time were $4{,}73\times10^3 \pm 0{,}5$ RNA copies / mkl for Schmallenberg virus and $6{,}57\times 10^2 \pm 0{,}5$ RNA copies / mkl for Akabane virus.

The designed assay is suitable to detect Schmallenberg and Akabane virus sequences in blood samples and organs of sheep and cattle. In contrast RNA from heterologous viruses or RNA extracted from normal samples have not produced positive results. This proves the specificity of the primers and the probes included in the assay. The analytical sensitivity of the assay was $23{,}65\times10^3 \pm 0{,}5$ copies of RNA per reaction for Schmallenberg virus, and $32{,}85\times10^2 \pm 0{,}5$ copies of RNA per reaction for Akabane virus.

Detection and molecular characterisation of equine infectious anaemia virus field isolate in the Omsk region of Russia.

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Introduction

Equine infectious anemia (EIA, swamp fever) is a contagious viral disease of equines. It is characterized by intermittent fever, anemia, loss of weight, and dysfunction of the cardiovascular system

The causal agent of equine infectious anemia is an RNA virus of the genus Lentivirus of the family Retroviridae..

EIA virus causes a clinical disease and persistent infection. Virus patogenicity has been shown for horses, ponies, donkeys and mules. EIA is worldwide distributed and was reported in Germany, France, Italy, Japan, Canada and others.

For the diagnosis of EIA before seroconversion the most widely used method is PCR.

The aim of this work was to detect EIA viral sequences using molecular-biological methods and genetically characterize EIA viruses isolated in the Russian Federation.

Materials & methods

50 samples of horse serum and 6 samples of blood were examined in this study. For diagnostic and sequence studies we used nested PCR. Extraction of viral RNA was performed using a modified method of Boom et al. Reaction for identification of the virus genome was carried out on PalmCycler device. The results of electrophoresis were visualized by using system Bio-Rad. For analysis of primary sequence of amplicons we used Applied Biosystem Genetic Analysis 3130 with a range of BigDye Terminator kit.

Results

In this report, a diagnostic study on EIA in naturally infected horses is presented. We investigated samples of horse blood and serum obtained from Omsk region farms of the Russian Federation. In this farms, seropositive horses were detected by agar gel immunodiffusion (AGID) [1]. Further in our laboratory several samples were tested by PCR.

For diagnostic of EIA in our institute were developed classical nested RT-PCR and Real time RT-PCR. In the present studies we used nested RT-PCR. Reactions were carried out using two different sets of primers targeting the *gag* region of EIA virus genome.

We have found eight positive samples containing the EIA virus sequences.

Amplicons of 8 PCRs were subjected to direct DNA sequencing and phylogenetic analysis. The obtained sequences were compared with genome published nucleotide sequences presented in the GenBank database using the program Blast.

Sequencing of parts of *gag*-gene showed that sequences here are closely related to those of European strain and isolates.

Nucleotide sequence homology was 83% with «EIAVGER-7» virus isolate, 80% with «Rom-4» virus clone and 79% - with «Ita90» virus isolate.

Discussion & conclusions

Using molecular biological techniques (PCR, sequencing) established the presence of EIAV in the samples from the Omsk region of Russia.

Sequense comparison established that Omsk EIAV isolates are similar to European isolates.

References

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New diagnostic tools for African Horse Sickness and Equine Infectious Anemia viruses control

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African Horse Sickness (AHS) and Equine Infectious Anemia (EIA) are two highly infectious vector-borne viral diseases of horses, including in the OIE listed diseases. The symptoms can be similar and their differentiation is significant. AHSV is endemic to Sub-Saharan Africa but sporadic outbreaks have had devastating effects in Northern Africa, Europe, and Middle East. Despite the low rate of incidence of EIA in Europe and USA, most countries require a negative test result before allowing an imported horse into the country. The increasing concerns for re-emerging diseases and the recent epidemiological situation with other vector-borne diseases, have highlighted the need for rapid and reliable tests for diseases control.

To this end, INGENASA has been working in the development and standardization of Lateral Flow Assays (LFA) as a rapid and easy diagnostic tool. The first aim of this study was to develop an immunoassay for the simultaneous serological detection and differentiation of AHSV and EIAV antibodies (Abs) in serum or plasma. Moreover, a LFA for AHSV direct detection has also been set up for serum, blood and tissue samples.

The prototype of the **duplex LFA AHSV/EIAV for Abs detection** is based on the VP7 and rp26 proteins from AHSV and EIAV, respectively. Different colored latex microspheres covalently linked to a control protein and to the corresponding viral antigens are used in the test. In the case of a positive sample, the antibodies will be captured by the antigen conjugated to the latex beads and later by the same specific target protein printed on the membrane. Depending on the type of antibodies present in the sample, different coloured lines will appear. On the other hand, the **LFA for AHSV detection** is based on the use of a MAb against AHSV VP7 protein. The same antibody is used both for conjugation to the latex beads and to the nitrocellulose membrane.

The initial results showed that the duplex LFA AHSV/EIAV was able to detect the 9 AHSV serotypes when using reference sera. In sera from horses experimentally infected with different AHSV serotypes, the test was able to detect antibodies between day 7-10 pi.

Besides, no cross-reactivity with other Orbiviruses was found, when the test was performed with sera form animals infected with either BTV or EHDV. Only a weak positive reactivity was shown with BTV serotype 17.

Preliminary results, reported good data of diagnostic sensitivity and specificity when field samples were analyzed. EIAV positive field horse sera from Argentina were tested on the LFA AHSV/EIAV with 100% of sensitivity.

Regarding the LFA for AHSV detection, first experiments with recombinant Ag and tissue culture virus were analyzed showing good data of sensitivity and specificity. Besides, no cross-reactivity was detected with other orbiviruses (EHDV/BTV).

Even though more samples need to be tested, these preliminary results suggest that the newly developed duplex LFA provides a reliable method for rapid detection of anti-AHSV and anti-EIA antibodies and AHSV. These two assays can be performed on the field providing useful tools in situations where laboratory support and skilled personnel are limited.

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An inherent decision support aid for the interpretation of diagnostic measurements

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Any decision about the presence or absence of a certain disease, which is made on the basis of diagnostic measurements, is of probabilistic nature. The use of a cut-off, which separates the population into two disjoint classes, i.e. ‚healthy' and ‚ill', based on result of the measurement process, is dissatisfying for two reasons: First, this classification may not reflect the truth and secondly, the distance of the measured value from the cut-off is not reflected in the statement. In particular, the theoretical distributions of the measurements, which form the basis for the distances between the cut-off and the individual measurement values, contain important information. This information provides the probability whether the decision based on the measurement value reflects the truth. It is important to note that this information does not affect the interpretation of the decisions based on the measured values nor their probabilistic character. It is therefore irrelevant if the classification decision relates to an individual or a population.

Normalized diagnostic values measured (e.g. OD in ELISAs or ct-values in quantitative realtime PCRs), which are based on clearly defined laboratory protocols, are nowadays generally highly accurate and thus reproducible compared to diagnostic approaches in earlier days. This allows interpretation of individual measurement values in a probabilistic way from the beginning, rather than assigning them to the classes ‘healthy’ or ‘ill’ in a fixed manner without considering all available information. As a result, the class affiliation ‘healthy’ or ‘ill’ is supplemented with the quantitative estimate of the certainty of each of these decisions. We therefore propose a decisional or epidemiological assessment of the health status of an individual or a population is therefore suggested, whereby the specific standardized individual measurement values serve as background information for the assessment of the health status. Two probabilities are assigned to each measured value, that is the probability that the measured value is a realization of the random variable based on the set of truly healthy and the probability that the same measured value is a realization of the random variable based on the quantity of truly ill individuals. Based on this approach, diagnostic measurements of increasing values correspond to an increasing probability of a correct classification as being ‘ill’ and thus a decreasing the probability of being ‘healthy’. This approach allows to avoid setting a particular cut-off. Instead of such a cut-off, two given probabilities exist for the possible disjoint decision of either ‘healthy’ or ‘ill’. These probabilities are only triggered by the characteristics or performance of the diagnostic test.

Validation of the ID Screen® FMD Type O Competitive ELISA

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Introduction

The ID Screen® FMD Type O Competition ELISA specifically detects antibodies against FMDV serotype O. It can be used with serum or plasma from cattle, swine or other susceptible species. This study summarizes validation data obtained for this ELISA.

Methods and Results

Analytical sensitivity: 8 bovine sera from Argentina, vaccinated with a trivalent FMD vaccine (serotype O, A and Asia 1), were serially-diluted and tested in parallel using the ID Screen® ELISA and commercial Kit A. The ID Screen® ELISA showed equivalent or superior analytical sensitivity to Kit A.

Exclusivity: The following commercially-available sera were tested in parallel using the ID Screen® ELISA and Kit A:

- 6 FAO / IAEA positive bovine sera from cattle infected with different serotypes
- 11 reference sera for different serotypes / strains from the Pirbright Institute

The ID Screen® ELISA correctly identified the serotype O sera as expected. No cross-reactions with other serotypes were observed for these panels, except for the SAT1 serum, which was found positive by both kits.

Specificity: The following sera from disease-free, certified herds were tested:
- 248 naïve cattle sera (France); measured specificity: 100% (CI $_{95\%}$: 98.47 - 100%);
- 263 naïve goat sera (France); measured specificity: 99.62% (CI $_{95\%}$: 97.88 – 99.93%);
- 210 naïve sheep sera (France); measured specificity: 99.05 % (CI $_{95\%}$: 96.6 – 99.74 %);
- 248 pig sera (France) and 152 Iberian pig sera (Spain); measured specificity = 100% (CI $_{95\%}$: 99.05-100%).

Comparative specificity: 32 swine sera and 32 bovine sera from disease-free, certified French herds were tested in parallel using the ID Screen® ELISA and Kit A.
- Measured specificity for the ID Screen® ELISA= 100% (CI $_{95\%}$: 94.34-100%).
- Measured specificity for Kit A= 98.44% (CI $_{95\%}$: 91.67-99.72%).

Conclusion

The ID Screen® FMD Type O cELISA shows excellent test performance. The test is particularly rapid and easy-to-use. All reagents are supplied ready-to-use (not freeze-dried), and results are obtained in 90 minutes. External validation work has been performed on samples from infected regions (data available upon request). IDvet welcomes propositions for collaborative validation work on this disease.

Novel ELISAs for differentiated detection of antibodies against either PRRSV EU or US in oral fluid

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Background: In the Danish SPF system PRRSV surveillance is based upon the ability to differentiate between the American (US, Type 2) and the European (EU, Type 1) strain of PRRSV. Danish swine herds are declared either free from PRRSV or positive to either PRRSV EU or PRRSV US – or both strains. The blocking ELISAs used in this surveillance are only validated for serum. Based on the same antigens (supplied by B. Strandbygaard and A. Bøtner, National Veterinary Institute, Denmark) as in the blocking ELISAs, indirect ELISAs for PRRSV EU and US were optimized for analysis of oral fluid (OF) samples.
Materials and methods: Samples for validation were obtained from PRRSV positive and negative Danish herds in collaboration with Practitioners from Odder Svinepraksis. OF pen pools were collected by hanging a rope in selected pens. For comparison, blood was drawn from all pigs in each OF-sampled pen. A total of 2551 sera and 281 OF pools were sampled, representing pigs from 15-100 kg. All sera were tested in the PRRS blocking ELISAs used in the SPF surveillance, and these results were used as a gold standard for the novel OF indirect ELISA: A PRRSV-positive pen was defined as a pen with at least 50% pigs positive in the blocking ELISA.
Results: In the novel US OF ELISA, choosing a pen specificity of 0,97, and a cut off value of 84 (calibrated OD value), the herd sensitivity with 10 pens sampled and a within herd pen prevalence of 0,2 would be 0,83. Likewise in the EU OF ELISA, with a pen specificity of 0,97 and a cut off value of 219 (calibrated OD value), herd sensitivity would be 0,78. This implies that if you take 10 rope samples, i.e. sample 10 pens, in one herd, the herd specificity will be 0,74 for both ELISAs.
As expected, a slight cross reactivity was found between the EU ELISA and the US ELISA. However, use of the abovementioned cut offs results in a reasonable specificity towards the heterologous strain in the two ELISAs. Thus specificity to the US strain in the EU-positive herds, is 74% and specificity to EU in the US herds, is 90%.
Conclusion: Based on these data we will continue developing a test system for OF, that can be used as a supplement for the serum based surveillance of PRRSV EU and US in Danish swine herds.

Phase I/phase II serological diagnosis as a tool for the control of Q fever in cattle

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In human medicine the diagnosis of Q fever is mainly based on serology (IFAT): phase II antibodies are associated to the acute status while phase I antibodies to the chronic status. In Veterinary diagnosis none of the available immunological assays can differentiate between chronic and acute status even if the identification of acute/chronic shedders is a critical issue in the control of Q fever infection at herd level.
The aim of this study was the evaluation of a modified commercial ELISA kit (Chekit Q Fever, IDEXX Laboratories) for the anti-*C.burnetii* phase I and II antibodies detection as a possible tool to identify the shedder status in cattle.
Sera were collected in 4 dairy herds with confirmed Q fever cases of abortion or confirmed active infection circulation. A total of 99 animals were sampled 3 times (every 2 months) and classified on the basis of serological tests (ELISA and CFT) and PCR on individual milk in 5 groups:

1) NI-: non infected, seronegative, not shedder (n=26)
2) NI+: non infected, seropositive, not shedder (n=29)
3) CS: seropositive chronic shedder (shedder at 3 sampling) (n=12)
4) OS+: occasional seropositive shedder (n=20)
5) OS-: occasional seronegative shedder (n=12)

The groups 1 and 5 were confirmed as negative with the modified kit, consequently only the groups 2, 3 e 4 were considered for the evaluation. All the 297 samples were stored at -20°C until tested with the modified ELISA kit. The statistical analysis was carried out on S/P values, by means of a mixed effects linear model.
Differences were observed among groups, permitting to discriminate CS group (more relevant from an epidemiological point of view) from NI+ and OS+ groups (less relevant for the outbreak control). Overall, NI+ showed significantly lower S/P values with respect to CS and OS+. Considering the interaction between group and phase, in OS+, the S/P values of phase I resulted significantly higher than S/P values of phase II. Conversely, no statistical differences were observed between the two phases for NI+ and CS.

Diagnosis and investigations on PED in Northern Italy

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In Italy and likely in Europe, the last epidemic of Porcine Epidemic Diarrhoea (PED) affecting pigs of all ages was described on 2005-2006, when PED coronavirus was identified in 63 herds by electron microscopy, PCR and serology (1). Watery diarrhoea without mucus and blood was associated with a reduction of feed consumption. In farrowing-to-weaning herds, diarrhoea affected the sows and suckling piglets, and the mortality in piglets was up to 34%. In growers and fatteners the morbidity ranged from 20 to 80%, but there was either no mortality or it was very low. Thereafter a systematic diagnostic approach was implemented to screening the presence of PEDV in Northern Italy. Two immunoassays were developed, based on Monoclonal antibodies (MAbs) produced against the European CV777 reference strain. An ELISA MAbs based "antigen-capture" to detect anti-PEDV antibodies, was compared with the immune-peroxidase monolayer assay test (IPMA) by testing 296 samples from 11 commercial swine farms. IPMA test and ELISA demonstrated a very good agreement, suggesting the use of ELISA as useful test for serosurveys (2). A double antibody sandwich enzyme-linked immune-sorbent assay (DAS-ELISA) was developed and firstly compared with RT-PCR in the examination of 506 specimens (faecal samples and intestinal contents) collected during 2006–2007 from pigs originating from different farms located in the Po valley (3,4). The correlation between the two methods was higher when testing faecal samples (K = 0.97, 95% CI: 0.94–1.00) than testing intestinal samples (K = 0.62, 95% CI: 0.35–0.89). Such methods were then employed as screening tools in field surveys to evaluate the presence and circulation of PEDV in pig farms in the North Italy. During the period 2008-2014 a total of 1563 samples from clinical cases of pig enteritis were investigated and PEDV was diagnosed in 61 outbreaks. A total of 21 positive ELISA cases and 5 positive samples previously detected in 2007 were then confirmed by RT-PCR, using PEDV specific primers (4). The amplified products were sequenced to establish genetic relationship of the partial S1 gene of field strains, and to perform phylogenetic analysis. The nucleotide and amino acid sequences were aligned and compared to selected PEDV sequences available from the GenBank database. Phylogenetic trees (generated by the neighbor-joining method by MEGA 5).showed that PEDV strains were divided into two groups: the first comprised the PEDV isolates from 2006 to the beginning of 2009, while the second contained the isolates from mid-2009 to 2012. Finally, to determine the actual sero-prevalence, over 500 pig sera from 15 farms were checked for the presence of antibodies. On the whole these results indicate the endemic presence of "PEDV European-like strains" in Italy since its last important epidemic occurrence around ten years ago.

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Technical evaluation of pretreatment protocols for viral metagenomics of RNA viruses.

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The revolution in sequencing output facilitated by next generation sequencing (NGS) platforms allows the high resolution characterization of microbial communities (metagenomics). Viral metagenomics approaches are increasingly being used for viral discovery in various tissue or blood samples from diseased animals. Various approaches are described, using different methodologies to target viral sequences in a sample. These include strategies ranging from the enrichment of viral particles to direct sequencing of total nucleic acids in a sample followed by bioinformatics identification of viral sequences. In this study, we compared different viral metagenomics approaches in order to reveal their sensitivity and potential bias introduced by enrichment and amplifications steps and to find the most suitable strategy for RNA virus discovery in clinical samples.

Newcastle disease virus was used as model RNA virus. In a first experiment we spiked different viral concentrations (10^6, 10^5, 10^4 EID50/ml) in lung tissue. A viral metagenomic approach using virion enrichment, random amplification and NGS was performed each time with and without a ribosomal RNA removal step. As NGS platforms we compared GS Junior (454 sequencing, Roche) and MiSeq (Illumina sequencing). Ribosomal RNA removal was found to improve the virus identification power of the protocol for field samples significantly. The MiSeq sequencer was found to be more sensitive for viral identification, due its higher sequencing output.

In a second experiment we spiked 10^4 EID50/ml in both lung tissue and serum samples. We compared (1) the influence of pretreatment steps (centrifugation, filtration, nuclease treatment) to no pretreatment on the sample, (2) the influence of ribosomal RNA removal to no ribosomal RNA removal on the RNA extract, and (3) the influence of random PCR amplification to PCR-free direct sequencing of cDNA. All conditions were sequenced with the MiSeq sequencer. Metagenomic data analysis was still ongoing at the time of submission of this abstract.

Time is of the essence; rapid identification of Veterinary pathogens using MALDI TOF

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Rapid and accurate identification of microbial pathogens is a cornerstone for timely and correct treatment of diseases of livestock and fish. Over the past years MALDI-TOF mass spectrometry has been proven a valuable tool in the routine identification of microbial isolates, circumventing the time consuming conventional biochemical tests. The utility of the technique in diagnostic laboratory is directly related to the quality of mass spectra and quantity of different microbial species in the database. Since commercial MALDI-TOF spectral database providers mainly focus on human pathogens there is a need for improving the datasets in order to extend the applicability of the technique to the veterinary field. Here we report upgrading of a commercial MALDI-TOF database with the mass spectra of fish and mastitis pathogens as well as pathogens relevant for surveillance of diseases of wildlife.

Relevant veterinary pathogens from the genera *Aeromonas, Vibrio, Yersinia, Flavobacterium, Streptococcus, Micrococcus, Moraxella, Pasteurella*, and *Staphylococcus* were selected from DTU-VET reference collection. All bacteria were cultivated by standard procedures. Mass spectra were produced from freshly grown cultures after extraction with formic acid/acetonitrile and by using an Autoflex Speed instrument (Bruker Daltonics). The obtained spectra were analysed by using Flex Analysis software and Biotyper 3.1 (Bruker Daltonics). The identity of isolates was confirmed by established phenotypic tests or by 16S rDNA sequencing.

All of the obtained mass spectra were of sufficient quality to allow unambiguous differentiation of the tested bacteria so the local database was upgraded with the following species: *Aeromonas salmonicida* (n=3); *Vibrio anguillarum* (n=16), *Yersinia ruckeri* (n=3); *Flavobacterium psychrophilum* (n=7), *Streptococcus canis* (n=4), *Streptococcus bovis* (n=1) *Micrococcus luteus* (n=1), *Moraxella bovis* (n=1*), Moraxella bovoculi* (n=2), *Pasteurella aerogenes* (n=2), *Pasteurella canis* (n=2), *Staphylococcus chromogenes* (n=5) *Streptococcus agalactiae* (n=5).

The upgraded spectral database has been extensively evaluated for identification of fish pathogens (*Aeromonas*, *Vibrio*, *Yersinia* and *Flavobacterium*) and to less extent for identification of mastitis and pathogens of wildlife. In all cases, however, there was an apparent improvement of Biotyper scores for identification at the species level. Further work is underway to improve quality of the database and to extend the applicability of the technique to identification at the sub-species level (microbial typing).

Phylogenetic analyses of equine arteritis virus (EAV) in aborted fetuses of horses in Turkey.

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Equine arteritis virus (EAV) is the aetiological agent of equine viral arteritis (EVA), a viral disease of the equide family which has been reported in many countries. Clinical signs of EVA include fever, depression, loss of appetite, conjunctivitis, nasal discharge, edema of the ventral body and limbs, abortion, and death in neonatal foals. Transmission occurs primarily by the respiratory route and venerally through infective semen. The aim of this study was to detect the RNA of Equine Arteritis Virus (EAV) in aborted fetuses of horses in Turkey by RT-PCR. In this study, 60 internal organs were collected from the aborted fetuses of horses from the Marmara region (bordering EU) of Turkey in 2010-2013. After homogenisation of the tissue, the viral RNA was extracted by using a commercial RNA extraction kit. RT- PCR assay was used to analyse test samples for the presence of equine arteritis virus (EAV) using a sequencing primers for EAV. The *"pol gene*" of EAV was partially sequenced and then phylogenetic analysis was performed. In conclusion; this study shows that EAV should be considered in aborted fetuses of horses in Turkey when investigating the cause of abortions.

Detection and Phylogenetic Analyses of Peste des Petits Ruminants Virus (PPRV) in Sheep in the Marmara Region of Turkey

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Peste des petits ruminants (PPR) is a highly contagious, notifiable and economically important viral disease of ruminants and has been reported in many countries including Turkey. The aim of this study was to investigate frequency, isolation, molecular detection and phylogenetic analyses of peste des petits ruminants virus (PPRV) in sheep and cattle in the Marmara region of Turkey. For this, blood, nasal swabs and lung samples from sheep and cattle were collected from the Marmara region of Turkey, between 2011 to 2012. Sera were analysed for the presence of antibodies to PPRV by using C-ELISA. Vero and Vero.DogSLAMtag cells were used for virus isolation. All samples were analysed by real-time RT-PCR using a TaqMan probe. The positives found by real-time RT-PCR were analysed by RT-PCR for sequencing using the primers specific to F and N genes of the PPRV. Out of 111 unvaccinated sheep and 50 cattle tested, antibodies to PPRV were detected in 13 sheep sera while all cattle sera were found to be negative for antibodies to PPRV. The viral growth was detected in both Vero cells and Vero.DogSLAMtag cell line by observing CPE (7 days) and the presence of PPRV in cultured cells was detected by real-time RT-PCR. These isolates were from the lung samples taken from two sheep originated from Istanbul. PPRV was detected in 22 (10.42 %) of 211 sheep samples (100 lungs and 111 blood and nasal swabs) by real-time RT-PCR. 9 of 22 samples detected by real-time RT-PCR could be amplified for sequencing by RT-PCR. The sequences were aligned with published sequences of PPRV and phylogenetic analyses have shown that strains detected in this study were clustered in lineage IV. **In conclusion,** PPR is still present in Turkey and effecting sheep health. The strains detected in this study clustered in lineage IV. The newly designed primers targeting the F gene in this study can be used in future analyses.