



Short communication

Seroprevalence of *Mycoplasma hyopneumoniae* in sows fifteen years after implementation of a control programme for enzootic pneumonia in Switzerland

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ARTICLE INFO

Keywords:

M. hyopneumoniae
Switzerland
Serology
Antibodies
Domestic pigs
Swine
Enzootic pneumonia
Control
Wild boar

ABSTRACT

Mycoplasma hyopneumoniae is the etiological agent of enzootic pneumonia (EP), an economically important chronic respiratory disease in pigs. *M. hyopneumoniae* impacts the mucociliary clearance system by disrupting the cilia and modulates the immune response, resulting in intermittent dry non-productive cough. For progressive control of EP in Switzerland, a corresponding programme was fully implemented in 2004. It is based on total depopulation strategies of affected fattening farms as well as partial depopulation in breeding farms. Surveillance of EP status in Switzerland is mainly based on real-time PCR of nasal swabs from coughing animals or suspicious lungs and thereby sporadic cases are still observed every year. In order to obtain information on the seroprevalence, serum samples of 5021 sows from 968 farms collected in 2018 at eight different slaughterhouses were analyzed for the presence of *M. hyopneumoniae*-specific antibodies using a commercial ELISA kit. The overall seroprevalence was low with 0.98% of sows testing positive and these seropositive animals could be allocated to 3.92% of farms tested. Most seropositive farms presented weakly positive singleton reactors and only one farm showed several strongly seropositive animals. In conclusion, the serological status mirrors the successful progressive control of *M. hyopneumoniae* in the Swiss domestic pig population over the years. The current study underlines the added value of serological testing in the surveillance of EP in a country with low prevalence and confirms the sustained benefit of strategic control programmes.

1. Introduction

Enzootic pneumonia (EP), a porcine chronic respiratory disease, is caused by the bacterium *Mycoplasma hyopneumoniae*. In infected animals, *M. hyopneumoniae* is mainly localised on the mucosal surface of the trachea, bronchi and bronchioles, where it affects the mucociliary clearance by ciliostasis and cilioatrophia, and modulates the immune system of the airways (Kuhnert and Jores, 2020; Maes et al., 2018). *M. hyopneumoniae* infection occurs through direct and indirect contact or airborne transmission (Leon et al., 2001). The clinical signs are dominated by an intermittent dry non-productive cough which can be

variable in intensity and last from weeks to months (Sibila et al., 2009). Most frequently affected by EP are growing and finishing pigs. EP incurs major financial losses due to reduction in growth performance, increased usage of antibiotics and the fact that it favours secondary infections (Maes et al., 2008). Besides domestic pigs, wild boars can also harbour *M. hyopneumoniae* (Kuhnert et al., 2011; Sibila et al., 2010).

Isolation of *M. hyopneumoniae* is cumbersome since its cultivation requires specialised media and its long division time favours overgrowth by other contaminating bacterial species. Therefore, PCR detection has become the method of choice in many countries, enabling swift diagnosis using clinical specimens, typically nasal (Zeeh et al., 2005) or

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<https://doi.org/10.1016/j.vetmic.2022.109455>

Received 1 February 2022; Received in revised form 13 April 2022; Accepted 9 May 2022

Available online 11 May 2022

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bronchial swabs (Dubosson et al., 2004; Stärk et al., 2007). In addition, serological tests such as enzyme-linked immunosorbent assay (ELISA) are widely used to detect *M. hyopneumoniae*-specific antibodies (IgG), but the seroconversion can take up to several weeks after infection (Fano et al., 2005; Leon et al., 2001).

Several commercial vaccines against *M. hyopneumoniae* are available, but they still have shortcomings (Maes et al., 2021) as most mycoplasma vaccines, essentially offering only partial efficacy and short duration of immunity. In addition, in the absence of a DIVA vaccine, commercial serological tests do not distinguish vaccinated from infected animals (Erlandson et al., 2005).

Switzerland implemented a programme for systematic control of enzootic pneumonia in 2004. For identification of diseased farms, a "mosaic diagnosis" was applied, which was built of clinical, laboratory and epidemiological datasets. Clinical or epidemiological suspicions were followed up by a cantonal veterinarian, who collected a minimum of three lungs, 10 nasal swabs or 20 blood- or colostrum samples, which were investigated by an authorised accredited laboratory in due course. In addition, in Swiss slaughterhouses, porcine lungs were examined for pathomorphological changes indicative of EP and if at least 10% of the pig lungs were found to show typical lung lesions, a real-time PCR-based protocol was used for direct detection of *M. hyopneumoniae*. In case *M. hyopneumoniae* was detected in domestic pigs, a total depopulation in fattening farms or a partial depopulation in breeding farms followed as a strategy for enhanced control of EP (Stärk et al., 2007). Vaccination was forbidden in the context of the control programme ever since 2004. Despite all the measures taken, sporadic cases of EP are still reported in Switzerland. This study aimed to investigate the seroprevalence of *M. hyopneumoniae* infection in Swiss domestic sows 15 years after initiation of enhanced control of EP. Moreover, we mapped cases reported between 2004 and 2018, and seropositive farms identified in this study, against densities of domestic pigs and wild boars to get an idea about the possible involvement of these wild animals in sporadic cases observed over the years.

2. Materials and methods

2.1. Serum samples

Representative porcine serum samples were collected in the framework of a national surveillance programme for detection of freedom of porcine reproductive and respiratory syndrome (PRRS) and Aujeszky's disease (AK). Samples were collected randomly from individual sows in eight different abattoirs scattered around Switzerland from January to July 2018, with a variation in the number of animal samples ranging from one to 63 per farm. The samples originated from 968 different farms. Serum samples were subsequently stored in aliquots at -20°C at the Institute of Virology and Immunology IVI in Mittelhäusern until use. For the present study, 5021 bio banked aliquots, from the PRRS and AK national surveillance programme, were randomly selected and subjected to the study.

2.2. Laboratory analysis

The IDEXX Mhyo Antibody ELISA Kit (IDEXX Europa B.V., Netherlands) was used to measure serum antibodies against *M. hyopneumoniae* according to the manufacturer's instructions. Briefly, all samples tested were diluted forty-fold (1/40) by mixing 10 μl of serum with 390 μl of sample diluent. Each sample was tested in duplicate in the antigen-coated 96-well plate using 100 μl per well, including the positive and negative controls provided by the kit. The plate was incubated for 30 min at room temperature ($18\text{--}26^{\circ}\text{C}$) and then washed three times using the Tecan Hydroflex washer (Tecan Group LTD., Switzerland). After adding an anti-porcine horseradish peroxidase conjugate, the plate was incubated for 30 min and washed for a second time. After adding 100 μl 3,3',5,5'-tetramethylbenzidine substrate and

incubating for 15 min at room temperature, a stop solution was added. The results of the assay were then measured as an optical density (OD) value using the Tecan sunrise reader (Tecan Group LTD, Switzerland) at a wavelength of 650 nm.

Two external positive controls, one low and one high positive, were included in each assay. The low positive control was prepared using positive samples from Swiss pigs diluted sixteen-fold (1/16) with serum from specific pathogen-free pigs. The sample was diluted to the point where it was on the cut-off between positive and suspect interpretation in order to see the variation between the different ELISA plates in the kits. For the high positive control, an undiluted sample of a highly positive pig from Germany was used.

For validity criteria the mean value of the positive controls (PCX) and negative control (NCX), provided by the kit, were obtained by the average value of the duplicates. The assay was valid if $\text{PCX}-\text{NCX} \geq 0.150$ and $\text{NCX} \leq 0.150$; otherwise, the test was repeated. The relative level of antibodies in the sample was determined by calculating the sample to positive (S/P) ratio using the equation provided by the kit: $\text{S/P} = \frac{\text{Sample Mean} - \text{NCX}}{\text{PCX} - \text{NCX}}$. The sample was considered negative if $\text{S/P} < 0.30$, suspect with $\text{S/P} \geq 0.30$ and ≤ 0.40 and positive if $\text{S/P} > 0.40$.

2.3. Data analysis

The software LabControl (Ticono GmbH Hannover, Germany) was used to calculate the S/P value and to obtain the result of the analysis. Data to be analysed were retrieved directly from the Tecan sunrise reader (Tecan Group LTD, Switzerland).

The seroprevalence was defined as the proportion of positive serum samples (without suspect samples) divided by all tested serum samples, and the herd prevalence by positive tested farms divided by all farms tested. A farm was considered positive if at least one sample tested positive.

2.4. Geographical distribution of domestic pigs

The data on the distribution of domestic pigs in Switzerland were downloaded from the website of the Federal Statistical Office (atlas.bfs.admin.ch/maps). The geographical data associated with the farms investigated in this study were obtained from the Federal Food Safety and Veterinary Office based on the Animal Tracing Database. The data on official EP cases from 2004 onwards were downloaded from the website of the Food Safety and Veterinary Office (infosm.blv.admin.ch).

If coughing occurs in a Swiss pig farm and EP is suspected, at least 10 coughing animals have to be sampled using nasal or tracheal swabs. EP cases according to Swiss legislation require positive real-time PCR results of the latter samples. Moreover if $> 10\%$ of lungs at slaughter show pathomorphological lesions typical for EP, at least three lungs have to be investigated by real-time PCR and if one is positive it is also a case. Optionally, at least three seropositive serum samples out of 20 serum samples tested confirm a case. In addition, such diagnostic results should always be accompanied with clinical or epidemiological data.

2.5. Geographical occurrence of wild boars

The relative abundance of wild boar per square kilometre in Switzerland was averaged over the years 2011–2018. It was computed using the hunting statistics, the beech mast index to balance hunting success and the probability of wild boar occurrence. A seven years period was considered to balance out the effects that non-controllable factors (e.g., weather) may have on hunting-success in a given year (Vargas Amado et al., 2021).

2.6. Spatial analysis

Maps were created using ArcMap 10.4 by ArcGIS® software

developed by Esri. Three types of data were used for the maps: tabular, raster and feature data. The raster corresponds to a Digital Elevation Model, which was used merely for visualisation purposes. The data for its creation was obtained from NASA (NASA/METI/AIST/Japan Space-systems and U.S./Japan ASTER Science Team. ASTER Global Digital Elevation Model V003. 2019, distributed by NASA EOSDIS Land Processes DAAC, <https://doi.org/10.5067/ASTER/ASTGTM.003>. Accessed 2021-10-19) where 15 images were downloaded, assembled and adapted to the shape of Switzerland. The tabular data corresponded to the number of domestic pigs per district (2018), the reported outbreaks per municipality (2004–2018) and all the tested farms. To contextualise these data in space, it was needed to pair them with spatial data. We downloaded geo-data from Swisstopo (Districts/Municipalities: District and Municipal boundaries, swissBOUNDARIES3D (swisstopo), 2018) which matched the spatial characteristics that the tabular data was referring to (e.g. districts or municipalities). The water bodies added to the figures were also taken from Swisstopo (Water bodies: Das Topografische Landschaftsmodell TLM 2021, swissTLM3D (swisstopo), 2021.).

3. Results and discussion

The aim of this study was to determine the seroprevalence of *M. hyopneumoniae* in domestic pigs 15 years after completion of a systematic control programme of enzootic pneumonia in Switzerland. Out of 5021 random serum samples tested, 49 samples derived from 38 farms reacted positive resulting in an overall prevalence of 0.98% (Fig. 1). 24 samples derived from 24 farms reacted as suspect (Fig. 1). Only one farm of the 968 farms enrolled in this study was remarkable with 10 positive samples out of 16 samples tested, many of them strongly positive (S/P 77 – 225%) indicating an ongoing infection with *M. hyopneumoniae*. The two external positive controls used in this study had S/P values above the positive cut-off value (>40%) in all plates tested, with mean values of 152% and 47%, respectively. The coefficient of variation between plates was found to range between 7.9% and 11%, which is similar to the one reported for the IDEXX test kit.

The overall seroprevalence of 0.98% found in this study is far below the seroprevalence in endemic countries (Pieters et al., 2020). For example, north west Germany was reported to have a seroprevalence of 65% in sows (grosse Beilage et al., 2009), and subtropical southern China a seroprevalence of 54.5% (He et al., 2011). The overall between-herd prevalence in this study was 3.92%, given that at least one animal per farm was tested positive. We expected seronegativity in the Swiss domestic pig population, based on the existing control programme implemented in 2004. Since vaccination and import of vaccinated pigs into Switzerland was and is forbidden, we can exclude seropositivity derived from vaccination in Switzerland (Stärk et al., 2007). The ELISA used in this study has a specificity of 97.2% on herd level (grosse Beilage et al., 2009) to 98.82 on animal level (Poeta Silva et al., 2020), which

theoretically could result in 2.8% of false-positive herds or 1.18% of false-positive animals. Although these numbers match the number of positive herds or animals in this study, false negative results cannot be assumed in the absence of means of verification. Pigs can be colonised by other *Mycoplasma* species, such as *M. hyosynoviae*, *M. hyorhinis* and *M. flocculare* (Gomes Neto et al., 2014; Petersen et al., 2016; Thacker and Minion, 2012). Especially *M. flocculare*, a commensal of the porcine respiratory tract, is likely to be present in domestic pigs, but information on its prevalence in Swiss domestic pigs is currently missing. Several studies investigating levels of cross reactivity of the IDEXX ELISA with other mycoplasmas than *M. hyopneumoniae* have been published (Erlandson et al., 2005; Gomes Neto et al., 2014). In a recent work investigating 50 individual mycoplasma antigens (Petersen et al., 2019), the authors did not find a difference in the antibody-reactivity between *M. flocculare* and *M. hyopneumoniae*. In that study, the proteins used were selected based on their membership in the P97/P102 gene families or structure predictions as being membrane proteins (lipoproteins). Therefore, samples that reacted weakly in our analysis could also be a result of cross reactivity with antibodies mounted against *M. flocculare* (Gomes Neto et al., 2014; Petersen et al., 2019).

In the Swiss control programme for EP, the IDEXX ELISA is only recommended for herd-based testing and not for testing of individual animals, because of its known low sensitivity in detection of not acutely-affected herds (Erlandson et al., 2005; Poeta Silva et al., 2020) combined with a specificity below 100%. The technical instructions of the Federal Food Safety and Veterinary Office require the analysis of at least 20 serum samples per herd. Seropositivity of a farm in the Swiss context requires at least three positive serum samples to enact epizootic consequences such as partial or full depopulation. Freedom of disease, not freedom from the pathogen is the focus.

In this study with random sample selection, only eight out of 38 seropositive farms met the criteria of 20 or more serum samples analyzed per farm resulting in a reduced power of detection. One breeding farm of these 38 farms tested positive in accordance with Swiss regulations and presents therefore a previously undetected EP case. This farm was located in the north-eastern part of Switzerland (Fig. 2D) and is likely to serve as a reservoir for *M. hyopneumoniae* and potentially infect other farms via animal translocation. All other farms tested positive did not meet the requirements according to the Swiss regulations. Additional results for evaluation of the serological results from direct pathogen detection, clinical and/or epidemiological indications of the sows tested in our study are not available since the metadata were anonymized.

The number of seropositive sows corresponds with the current EP situation in Switzerland, since sporadic cases are reported. An eradication programme in Norway was implemented in 1994 and included serological testing, where positive serum samples were reported during the 15 years of eradication until 2009 and since then Norway has been considered free of *M. hyopneumoniae*. Interestingly, the serological

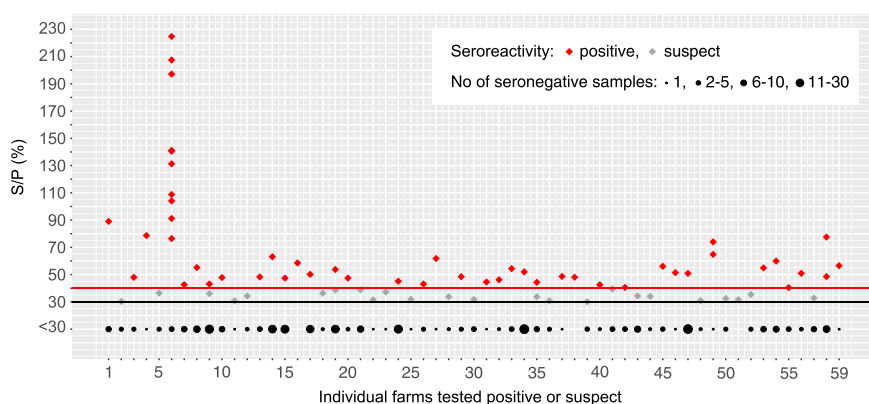


Fig. 1. Graphical representation of the 59 serum samples that reacted positive or suspect and the number of remaining serum samples of the same farms. Symbols in vertical line belong to one farm. Cut-off values for positive and suspect samples are represented by the red and black horizontal lines, respectively. The number of negative samples per farm that had positive or suspect reactors are displayed as black circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

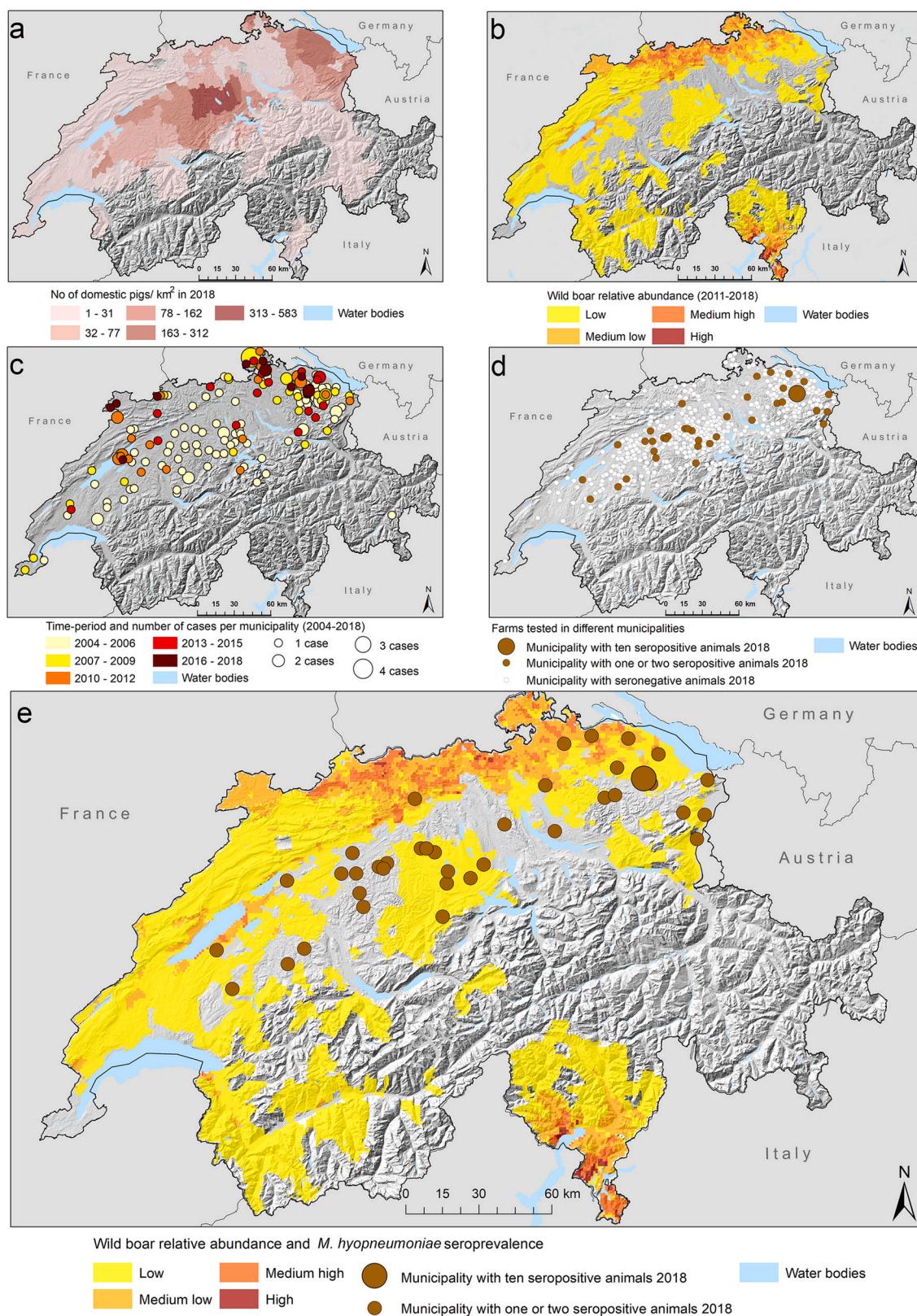


Fig. 2. Geographical maps of Switzerland displaying densities of domestic pigs, wild boars, reported cases of enzootic pneumonia and seropositive farms of this study. (A) domestic pig density (pigs/km²) of 2018, (B) averaged wild boar relative abundance calculated based on data retrieved between 2011 and 2018, (C) reported cases of EP since 2004, with time-period and number of cases per municipality, (D) municipalities in which the 968 farms tested in this study, using the random serum samples collected by the Swiss authorities in 2018, revealed seropositive and seronegative animals. (E) Overlay map showing the seropositive animals of this study and estimated wild boar populations.

testing in October 2006 already showed no known EP positive sow herds, but retesting in 2007 revealed several seropositive sow herds and one multiplier herd in one county being positive again. The Norwegian pig population and density is much lower than the Swiss domestic pig population. Since Norway was declared free from *M. hyopneumoniae* in 2009, positive serum samples were not detected in the 44,228 serum samples tested subsequently (Gulliksen et al., 2021).

In order to get an idea whether the pig density or the wild boar population may play a role in sporadic cases, we visually assessed the distribution of wild boars and domestic pigs in relation to previously confirmed EP cases and to seropositive farms determined in this study (Fig. 2). Fig. 2A shows the domestic pig density (pigs/km²) in 2018, with the highest densities located in the central and north-eastern part of the country. The wild boars, as determined between 2011 and 2018, have a high averaged relative abundance in the northern and north-western part of Switzerland on its border with Germany and France, and in the South on its border with Italy (Fig. 2B, C). In terms of EP, recent cases (2016–2018) were found in the north-western part on the border to France and in the north-eastern part on the border to Germany (Fig. 2C). This latter map shows also a decrease of cases over time since 2004. Finally, Fig. 2D shows the location of the 968 farms from which sera, collected randomly by the Swiss authorities in 2018, were tested in the present study. The geographical distribution of the farms tested seropositive for sows in the present study (Fig. 2D, E) corresponds approximately to the general distribution of reported cases in the past and does in part overlap with the wild boar population (Fig. 2E). Reported cases of EP in recent years have increasingly moved to the areas around the north-western and to a higher extent in the north-eastern parts at the Swiss border. In the latter region, this study revealed an unreported case of EP in one farm. In Switzerland's neighbouring countries, there is no control programme for EP. Data on seroprevalence in these bordering regions are therefore missing, while for instance in north west Germany, a seroprevalence of 65% was observed in sows (grosse Beilage et al., 2009).

Although the most common cause of an EP outbreak is the introduction of infected pigs (Maes et al., 2018), our study indicates a potential role of wild boars, since high densities of the latter have a similar geographical distribution as the cases found between 2004 and 2018 (Fig. 2B, C, E). The specifics of the Swiss domestic pig production system with a relatively large proportion of outdoor keeping favours interactions with wild boars. We know that Swiss wild boars carry classical outbreak strains as well as atypical *M. hyopneumoniae* strains (Kuhnert and Overesch, 2014) and that *M. hyopneumoniae* is widespread in Swiss wild boar populations (Batista Linhares et al., 2015), while the transmission of domestic pigs to wild boars seems to be most likely. To date, the potential of atypical strains to induce EP in domestic pigs is unknown, and we plan to investigate experimentally if these strains cause clinical disease or simply leave serological signatures after infection. If the latter is the case, it could explain the serological positive samples of this study. Overall, our study supports the progressive control of EP in Switzerland that resulted in a very low seroprevalence in sows compared to neighbouring countries such as Germany and Austria.

4. Conclusion

This serological study confirms that the control programme of EP in Switzerland was highly effective. Switzerland has progressively controlled EP without any vaccination. However, the pig production system in Switzerland differs from other pig-producing countries such as Germany or Denmark especially in terms of a much lower number of animals per farm. A DIVA type vaccine would be helpful to implement control programmes similar to Switzerland in other countries highly affected by EP at least for an initial control phase, since it would reduce the number of farms to be fully or partially depopulated.

Author contributions

JJ designed the study. NR helped with the logistics of the the serum samples. NS and GO did the serological analyses. NS, PK and JJ drafted the manuscript. NR and KS edited the manuscript. MEVA did the GIS maps. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This study was funded by the Federal Food Safety and Veterinary Office, Switzerland (FSVO Project No: 1.21.03) and supported by the University of Bern, Switzerland and the Institute of Virology and Immunology IVI, Switzerland. We thank the FSVO for providing the serum samples from their bio bank, Isabel Henning Pauka for the provision of seropositive control serum samples and Hatice Akarsu as well as Sergi Torres Puig for their help.

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