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ORIGINAL ARTICLE

Evidence for swine and human papillomavirus in pig slurry in Italy

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Abstract

Aims: The diversity and the geographical distribution of swine papillomaviruses (PVs) are virtually unknown. The occurrence and the diversity of swine PV were therefore investigated in pig slurry collected in Italy, to contribute towards filling this gap in knowledge.

Methods and Results: Twenty-two slurry samples underwent analysis by nested PCR and DNA sequencing using published and newly designed specific primer pairs for *Sus scrofa* papillomavirus (SsPV) type 1 and 2 (SsPV1 and 2), along with degenerate PV-specific primers targeting the major coat protein L1 and the helicase protein E1. Overall, three samples (13·6%) were positive for SsPV1 by specific primers, and nucleotide (nt) sequences showed 99–100% nt identity with SsPV1 variant a (EF395818), while SsPV2 was not found in any sample. Using generic primers, eight samples (36·4%) were tested positive for human papillomavirus (HPV), and were characterized as follows: β1-HPV8, β1-HPV14, β1-HPV206, β2-HPV113, β2-HPV120 and γ1-HPV173. Moreover, one unclassified γ-type was detected.

Conclusions: Both swine and human PVs were detected in pig slurry in this study. The unexpected presence of HPV in pig waste could be explained as the result of an improper use of the sewage collection pits and/or with improper procedures of the operators.

Significance and Impact of the Study: This study reports the first detection of SsPV1 in Italy, along with the first detection of HPVs in pig slurry samples in Italy, and expands our knowledge about PV diversity and geographic distribution.

Introduction

Papillomaviridae is a large family of viruses that infect the epithelium of skin and mucosa causing benign and malignant tumours in a wide variety of vertebrates such as bovines, canines, equines, lagomorphs, birds and humans. Phylogenetically, Papillomaviruses (PVs) group into 49 genera, each further divided into species and types (http://pave.niaid.nih.gov/). The rapid evolution of the PCR-based protocols and the high-throughput sequencing technologies has led to a continual discovery of new species and types (Arroyo *et al.*, 2013; Bzhalava *et al.*, 2014; Bzhalava *et al.*, 2015; Brancaccio *et al.*, 2018). The Papillomavirus Episteme database (PaVE) (van Doorslaer *et al.*, 2017), established to provide organized and curated PV genomics information and tools, currently contains 330 reference genomes of human PV (HPV) and 183 reference genomes of animal PVs (https://pave.niaid.nih.gov/#home, accessed on 14 March 2019). The number of characterized animal PVs increases

continuously (Mengual-Chulia *et al.,* 2012; Rector and Van Ranst 2013) to the extent that recently, the proposal to create an International Reference Center for Animal PVs has raised (van Doorslaer and Dillner, 2019) in addition to the Referent Centre for the human viruses (www.hpvcenter.se).

Currently, there are only two reference PV sequences within the Suidae family: *Sus scrofa* papillomavirus 1 (SsPV1), classified into the species *Dyodeltapapillomavirus* 1 (further divided into variants a and b) and *Sus scrofa* papillomavirus 2 (SsPV2), which is still unclassified. The first study on Swine PV was published in 2008 by Stevens and co-workers, who used degenerate primers to amplify novel PVs from the healthy skin of domestic pigs in Belgium and reported the complete genomic sequence of two variants: SsPV1a and 1b (Stevens *et al.,* 2008). Eight years later, in 2016, a novel PV, named SsPV2, was isolated and sequenced from wild boar in Germany (Link *et al.,* 2017). It was the first swine PV associated with a skin fibropapilloma. However, phylogenetic analysis was not able to relate SsPV2 definitely with other PV species or existing genera; therefore, it was suggested as the potential representative of a new PV genus (Link *et al.,* 2017).

Swine PVs have not been described in other parts of the world: currently, the swine sequences of the two mentioned studies are the only available in the GenBank database.

Since SsPV diversity and geographical distribution are virtually unknown, with only two studies published on the topic (Stevens *et al.,* 2008; Link *et al.,* 2017), in the present work we investigated the occurrence and the diversity of SsPVs in pig slurry in Italy using both swine specific and broad-range PV primers, known to detect both human and animal PVs.

Materials and methods

Twenty-two slurry samples were collected in 2015 from 10 farms placed in Northern Italy (Emilia Romagna and Lombardy Regions) in the framework of a previous study (La Rosa *et al.,* 2016). Briefly, 5 ml of slurry was diluted to 20 ml with sterile water and then treated with 2 ml of 2·5 mol l^{-1} glycine pH 9·5 and incubated in ice for 30 min. Samples were then washed with chloroform twice and centrifuged after of vigorous vortexing. Viral nucleic acids extraction from chloroform-treated samples took place using the NucliSENS easyMAG (BioMerieux, Marcy l'Etoile, France) semi-automated extraction system.

Genomes underwent analysis for swine and human PVs using nested PCR with published and newly designed primers targeting the L1 (major capsid protein) and E1 regions (helicase protein involved in viral replication). For the detection of SsPV1, the first PCR cycle foresaw the use of published primers (Stevens *et al.,* 2008) while newly designed primers were designed for the nested reaction. For SsPV2 detection, novel primer pairs (designed on the unique available sequence in GenBank) were used to amplify L1 and E1 fragments corresponding to those of SsPV1 primers. However, broad-range sets of primers were also used to detect both human and animal PV. These targeted the L1 (MY09/MY1-GP5/GP6, FAP59/64-FAP6085/6319, CP65/70-CP66/69, AR-L1F1/AR-L1R3 and AR-L1F8/AR-L1R9) and E1 regions (PM-A/PM-B) according to previously validated protocols for the detection of mucosal and cutaneous HPV genotypes (La Rosa *et al.,* 2013). Table 1 shows the list of primers and PCR used in this study, along with the amplicon length and the references.

PCR reactions with broad-range primers were performed in 25 μl of reaction mix using 2 μl of DNA and 1 μl (10 pmol) of primer, using the TaqGreen Mix PCR (Promega, Mannheim, Germany). Reaction conditions were as follows: 94°C (10 min) as first denaturation step, 45 cycles of: denaturation at 94°C (1 min), annealing at 50°C (1 min) and extension at 72°C (1 min) with a final extension step at 72°C (5 min). Nested PCR was performed using 2 μl of the first PCR product at the same conditions of the first amplification.

PCR reactions were carried out in a 96-well thermocycler (Applied Biosystems, Life Technologiess, Carlsbad, CA) and analysed on agarose gel (1·8%) electrophoresis stained with ethidium bromide. PCR products of expected length were purified by a Montage PCRm96 Microwell filter plate (Millipore, Burlington, MA) and subjected to direct automated Sanger sequencing on both DNA strands (Bio-Fab Research, Rome, Italy). The forward and reverse sequences were edited and assembled into contigs using MEGA software ver. 6.0, and consensus sequences were compared to prototype sequences of the PaVE (http://pave.niaid.nih.gov/#home) and of GeneBank database using the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/, to find the most related reference types.

Phylogenetic analysis was performed using MEGA software ver. 6.0. The alignment of nucleotide (nt) sequences took place using the CLUSTAL W algorithm. The phylogenetic tree was constructed using the maximum likelihood method based on the general time reversible model, integrated into the MEGA software.

Results

Samples were analysed using nested PCR with the published and newly designed primer shown above. High-quality DNA sequences were always obtained by direct

Table 1 Primers used in the present study for PCR and sequencing

Primer name	Region	Amplicon bp	Primer sequence 5′-3′	References
SsPV-L1F	L1	570 bp (first cycle)	CACGGACGAATATGTACAGC	Stevens *et al.* (2008)
SsPV-L1R			GTCCTGAAGTCCATGTTACC	
2158-SsPV-L1F-nest		414 bp (nested)	CGAATATGTACAGCGCACCC	This study
2159-SsPV-L1R-nest			GGTGTGCAGCCTAACATGAA	
SsPV-E1F	E1	574 bp (first cycle)	GTACAAGAAGGCTGAGATGC	Stevens *et al.* (2008)
SsPV-E1R			GTATACTGGGTCACCTTTGC	
2160-SsPV-E1F-nest		409 bp (nested)	AAGCAGGCGGACAGATATGA	This study
2161-SsPV-E1R-nest			TTAAGAGTGGTGGCCCCTTT	
2231- SsPV2-L1F	L1	458 bp (first cycle)	CTGTAACAGTTCCTAAGGTCTCT	This study
2232-SsPV2-L1R			GTCCATATTTCCAAAGCCGGT	
2231- SsPV2-L1F		435 bp (eminested)	CTGTAACAGTTCCTAAGGTCTCT	
2233-SsPV2-L1R-nest			CGGCCATATCCCCATCTTCTA	
2234-SsPV2-E1F	E1	501 bp (first cycle)	AGGCTGAAATGAATGAGATGTCT	
2235-SsPV2-E1R			TCAAATATGGCCACCTATCTTCT	
2236-SsPV2-E1F-nest		478 bp (nested)	GATGTCTACTGCTCGGTGGA	
2237-SsPV2-E1R-nest			TGGCCACCTATCTTCTTCCT	
MY11	L1	449 bp (first cycle)	GCMCAGGGWCATAAYAATGG	Manos *et al.* (1989)
MY09			CGTCCMARRGGAWACTGATC	
GP5+		138 bp (nested)	TTTGTACTGTGGTAGATACTAC	de Roda Husman *et al.* (1995)
GP6+			GAAAAATAAACTGTAAATCATATTC	
FAP59		484 bp (first cycle)	TAACWGTIGGICAYCCWTATT	Forslund *et al.* (1999)
FAP64			CCWATATCWVHCATITCICCATC	
FAP6085		238 bp (nested)	CCWGATCCHAATMRRTTTGC	Forslund *et al.* (2003a); Forslund *et al.* (2003b)
FAP6319			ACATTTGIAITTGTTTDGGRTCAA	
CP65		452–467 bp (first cycle)	CARGGTCAYAAYAATGGYAT	Berkhout *et al.* (1995)
CP70			AAYTTTCGTCCYARAGRAWATTGRTC	
CP66		377 bp (nested)	AATCARMTGTTTRTTACWGT	
CP69			GWTAGATCWACATYCCARAA	
AR-L1F1		599 bp (first and second cycle)	TTDCAGATGGCNGTNTGGCT	Rector *et al.* (2005)
AR-L1R3			CATRTC HCCATCYTCWAT	
AR-L1F8		704 bp (first and second cycle)	GGDGAYATGDGKGAMATWGG	
AR-L1R9			GGRCATTTKGTWGCWADGGA	
PM-A	E1	117 bp (first and second cycle)	ACTGACCAAAGCTGGAAATC	de Koning *et al.* (2006)
PM-B			TCTTGCAGAGCATTGAAACG	

amplicon sequencing. Table 2 reports the sequencing results: three samples (ID17, ID19 and ID20), collected from two different farms, tested positive with the SsPV1 L1 nested assay. Upon sequencing, they showed 99–100% nt identity with the GeneBank sequence EF395818, classified as SsPV1 variant a. Only one of these positive samples could be further amplified with primers targeting the E1 coding region, which resulted in 99% nt identity with EF395818. No amplification was obtained with the newly designed primers specific for SsPV2.

Eight samples tested positive using the broad-range FAP6085/6319 primers and one of these also proved to be positive with CP66/69 primers, while no amplicons were produced with the other tested oligos. All positive samples were characterized as human PVs. Six different types were identified: HPV8, HPV14, HPV206 (HPV-mRTRX7nr) (Betapapillomavirus 1), HPV113 and HPV120 (Betapapillomavirus 2), and HPV173 (Gammapapillomavirus 1). Moreover, one sequence could not be assigned to any classified HPV since the most related reference sequence in PaVE database was γ19-HPV166, with only 80·7% of nt identity (coverage 74·7%). However, it showed 99% nt identity with two unclassified γ-types in GenBank: isolate HPV-mSK_054 (MH777199) found in skin swab in DOCK8-deficient patients (Tirosh *et al.*, 2018) and isolate KC31 (KC752034) found in skin samples collected from healthy individuals (Li *et al.*, 2013).

One sample contained two HPV sequences: the amplicon obtained with FAP6085-6319 primers was characterized as β2-HPV113, while the amplicon found with CP65/70 primers was characterized as β2-HPV120.

Figure 1 depicts the results of the phylogenetic study, performed on the sequences obtained in the L1 region. The tree includes the sequences obtained in this study, in addition to the prototype sequences from the PaVE

Table 2 Sequencing results for swine and human PVs

Sample ID	Most closely related reference type in PAVE database	Nt identity	Most closely related sequence in GenBank	Nt identity
Swine papillomavirus				
ID17	SsPV1 (*Dyodeltapapillomavirus* 1); EF395818	100%	EF395818	100%
ID19		99%		99%
ID20		99%		99%
Human papillomavirus				
ID2	HPV113 (*Betapapillomavirus* 2); FM955842	95·1%	FM955842	95·1%
ID2	HPV120 (*Betapapillomavirus* 2); JQ963500	98·2%	JQ963500	98·2%
ID3	HPV14 (*Betapapillomavirus* 1);X74467	98·9%	X74467	98·9%
ID7, ID12, and ID23	HPV206 (*Betapapillomavirus* 1); U85660	98·1%	U85660	100%
ID8	HPV173 (*Gammapapillomavirus* 1); KF006400	91·2%	AF121431	100%
ID11	HPV8 (*Betapapillomavirus* 1); M12737	100%	M12737	100%
ID21	HPV166 (*Gammapapillomavirus* 19); NC_019023	80·7% (QC 74·7%)	MH777199 and KC752034	99%

QC, query coverage.

database and from GeneBank. Sequences from slurry samples are grouped into five different clusters corresponding to *Betapapillomavirus* 1 and 2, *Gammapapillomavirus* 1 and 19, and *Dyodeltapapillomavirus* 1, according to the genotyping results by BLAST analysis.

Discussion

Papillomaviruses affect all domestic animals and many wild vertebrates. This study reports the first detection of swine PV in Italy, specifically SsPV1 variant a. SsPV1 is the prototype virus for the species *Dyodeltapapillomavirus* 1, the single representative of the genus *Dyodeltapapillomavirus*, detected from swab samples taken from the healthy skin of two female domestic pigs in Belgium (Stevens *et al.,* 2008). Little is known about the diseases that SsPV causes. A few studies documented fibropapillomatosis in piglets and transmissible genital papilloma in pigs (Rang and Dimigen 1971; Rieke 1980; Vitovec *et al.,* 1999). However, these were before molecular techniques made it possible to sequence the genome and therefore, the infectious agent could not be characterized precisely. More recently, Nishiyama *et al.* (2011) examined a cutaneous fibropapillomatosis on the head and the back skin of a 4-month-old piglet, but PV antigen and DNA were not detected in these lesions by immunohistochemistry and PCR, therefore the fibropapillomatosis was considered hamartomatous rather than infectious. However, the generic MY09/11 primers used in that study might have failed to detect swine PV. In the present study, a wide panel of broad-range primers has been used, including MY09/11 and FAP primers, the latter previously proved to allow the amplification of different animal PVs (Antonsson and Hansson, 2002; Li *et al.,* 2013; Silva *et al.,* 2013). However, only specific primers for SsPV provided positive results. It is likely that esophytic or endophytic lesions such wart papillomas and venereal papillomas are more common than known in the swine population. Since animals with lesions are rapidly removed from the herds, the identification of PV is rarely performed. As consequence, the only available SsPV sequences in GeneBank and in PAVE database are the SsPV1 variants a and b (EF395818 and EF395819) found in healthy skin of domestic pigs (Stevens *et al.,* 2008), and the SsPv2 sequence (KY817993) found in a wild boar with esophytic papillomatosis (Link *et al.,* 2017).

In our study, positive results for SsPv2 were not found. However, the newly designed primers for SsPV2 were engineered on the basis of the unique available sequence in GeneBank, and we cannot be certain about the proper functioning of such primers since the genetic variability of SsPv2 is still unknown.

Seven HPV genotypes were detected in pig slurry samples: HPV8, HPV14, HPV113, HPV120, HPV173, HPV206 (mRTRX7nr) and an unclassified γ-type. Table 3 reports a summary of the clinical conditions associated with them.

In one sample, two different types were detected, demonstrating that slurry samples, like wastewater samples may contain multiple viral strains and can be therefore a good matrix to explore viral diversity.

The occurrence of human PVs in swine slurry was unexpected. It is conceivable that the presence of HPVs in pig waste might result from improper use of the sewage collection pits or from operators negligence to comply with the biosafety regulations. However, the possibility that human PVs infect and multiply in pigs cannot be excluded in the absence of specific studies.

In conclusion, this study reports the first detection of SsPV1 in Italy, along with the first detection of HPVs in

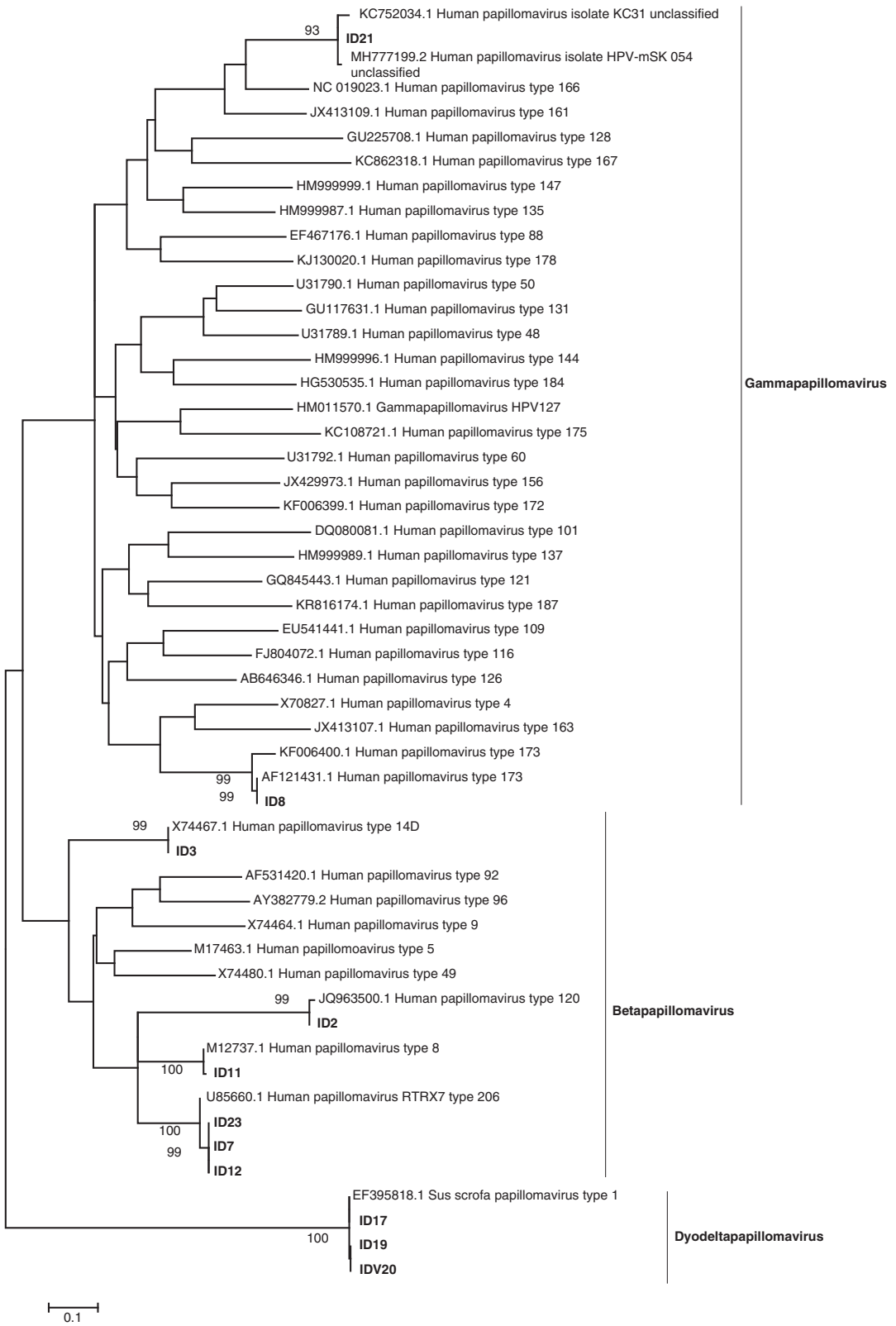

Figure 1 Phylogenetic tree constructed with the sequences obtained in the L1 region. The tree was constructed using the maximum likelihood method based on the general time reversible (GTR) model. The sequences identified in this study are in bold.

Table 3 Clinical conditions associated with the PVs detected in pig slurry in this study

PV type	Lesion type, isolation site and source	Main references
SsPV1variant a	Healthy skin of domestic pigs	Stevens *et al.* (2008)
β1-HPV8	Healthy skin, eyebrow hair, EV lesions, mouth, anal canal, AK, SCC	Bzhalava *et al.* (2014); Donà *et al.* (2019); Ekstrom *et al.* (2013); Fuchs *et al.* (1986); Rollison *et al.* (2019); Torres *et al.* (2015)
β1-HPV14D	Healthy skin, eyebrow hair, oral rinse samples, oral cancer, oropharyngeal cancer, EV lesions, anal canal, KS lesion, AK, KA, SCC	de Oliveira *et al.* (2004); Donà *et al.* (2019); Donà *et al.* (2015); Murahwa *et al.* (2014); Rollison *et al.* (2019); Sabol *et al.* (2016); Torres *et al.* (2015); Winer *et al.* (2018)
β1-HPV206	Healthy skin, SCC, AK	Hopfl *et al.* (1997); Tirosh *et al.* (2018)
β2-HPV113	Healthy skin, eyebrow hair, oral rinse samples, wart, anal canal, SCC, BCC, HNC, oral cancer, oropharyngeal cancer	de Villiers and Gunst (2009); Donà *et al.,* (2019); Donà *et al.,* (2015); La Rosa *et al.* (2013); Rollison *et al.* (2019); Sabol *et al.* (2016); Torres *et al.* (2015); Winer *et al.* (2018)
β2-HPV120	Healthy skin, eyebrow hair, oral cavity, SSC, stool samples, anal canal oral cancer, oropharyngeal cancer	Bottalico *et al.* (2012); Donà *et al.* (2019); Donà *et al.* (2015); Ma *et al.* (2014); Rollison *et al.* (2019); Sabol *et al.* (2016); Torres *et al.* (2015); Winer *et al.* (2018)
γ1-HPV173	Healthy skin; oral rinse samples, eyebrow hair, HNC, SPA/pool	Di Bonito *et al.* (2017); Martin *et al.* (2014); Rollison *et al.* (2019); Winer *et al.* (2018)
Unclassified γ-type	Healthy skin	Tirosh *et al.* (2018)

SCC, squamous cell carcinoma; AK, actinic keratosis; BCC, basal cell carcinoma; KA, keratocanthoma; HNC, head and neck carcinoma; EV, epidermodysplasia verruciformis; KS, Kaposi sarcoma.

pig slurry, fostering the need of further studies to expand our knowledge about swine PV geographic distribution dynamics. The results of the present study suggest that these PVs could be more widespread than previously considered. Also, swine PV diversity needs to be studied: the lack of sequence information, with only three SPV sequences currently available, hampers the design of novel PCR primers capable to successfully detect SPVs. The next-generation sequencing in swine and slurry samples could open new avenues to exploring SPV diversity.

The potential risks for humans linked to the presence of swine and human PV in pig slurry are still unknown. Although PVs are host-restricted, the cross species transmission may also occur albeit rarely (Nasir and Campo 2008; Silvestre *et al.,* 2009; Gottschling *et al.,* 2011; Roperto *et al.,* 2013; Bravo and Felez-Sanchez, 2015), and the interspecies transmission of viruses (e.g influenza and Coronavirus) involving pigs have been demonstrated (Yassine *et al.,* 2013; Rajao *et al.,* 2018). As for human PVs, their presence in pig slurry opens issues on their potential harmfulness for humans with particular reference to both occupational health risks for animal farmers (because of the direct contact they have with pigs and their wastes, e.g. during the periodical cleaning barns undergo) and the potential environmental pollution resulting from manure spreading on the soil. As a matter of fact, Ngabo *et al.* (2016) pointed out that, being farmer was among the secondary significant risk factors for HPV positivity (besides HIV-positivity) in the population they studied, even though they do not relate this with the exposure to farm effluents (Ngabo *et al.,* 2016).

The seasonal temperature transitions manures experience has slight or negligible effects on manure characteristics and virus persistence (Auffret *et al.,* 2019). However, acting on chemical manure properties may represent a potential improvement. Increasing manure pH, on the one hand, affects virus persistence (Hurst *et al.,* 1980; Stevens *et al.,* 2018) but, on the other, it causes ammonia emission from manures into atmosphere to increase (Petersen and Sommer, 2011) so that it cannot be considered among the best available techniques recommended for pig rearing (Giner Santonja *et al.,* 2017). Therefore, further investigation of HPV dynamics in manures could allow the set-up of effective in-farm manure management methods and practices aimed at improving the safety of exposed workers and lowering virus persistence in farm effluents and soils, in compliance with environmental and rearing regulations.

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Conflict of Interest

The authors declare that there are no conflict of interest.

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