DETECTION AND SUBTYPING OF SWINE INFLUENZA VIRUS BY RT-PCR AND STANDARD METHODS

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Introduction

Influenza virus causes outbreaks of acute respiratory disease in man and animals; three virus types (A, B, C) can be distinguished on the basis of antigenic differences of nucleoprotein (NP) and matrix (M) proteins. Influenza A virus infects a wide range of avian and mammalian species. Type A viruses are further divided into subtypes, based on the antigenic nature of their surface glycoprotein haemoagglutinin (HA) and neuraminidase (NA). Ducks and other waterfowl birds are the principal natural hosts of influenza A virus (10). From this natural reservoir viruses are transmitted to other species; among these an important role in influenza epidemiology is held by swine (8). Swine influenza virus (SIV) causes serious economic consequences because of the increased time needed for infected animals to obtain slaughter weight. SIV is a zoonosis for which pigs may act as intermediate host and mixing vessel for genetic reassortment between human and avian viruses (8). The passage of influenza A virus from animal host to man may lead the emergence of new pandemic strains; the prompt detection and identification of such events are paramount in the surveillance of influenza viruses. In Europe three major influenza A subtypes (H1N1, H1N2, H3N2) actually circulate in swine population. Diagnosis of SIV includes application of various methods. Isolation by inoculation of fertilized chicken eggs with pathological samples is considered as "gold standard" method (7). Cell culture system showed to be a reliable substrate for influenza virus replication (3, 6). Viral isolation and identification by eggs or cell culture inoculation are however time consuming methods. To have more rapid results in virus influenza detection, rapid enzyme immunoassay tests (9) and RT-PCR assays could also be used (2). Antigenic characterization of influenza A virus isolates is traditionally performed by serological tests, Haemoagglutination Inhibition (HI) and Neuroaminidase Inhibition (NI) tests. Application of diagnostic techniques based on molecular studies recently has supported serological tests by development of two RT-PCR assays to detect and subtype swine influenza virus (SIV). The aim of this study is to compare standard virological methods, eggs and cell culture inoculation, with RT-PCR based on the matrix (M) gene and to compare two multiplex RT-PCR methods for subtyping SIV isolates with HI test.

Materials and methods

Samples. 441 pathological samples (lung, nasal and tracheal swabs) were collected between June 2001 and December 2002 in 177 outbreaks of respiratory diseases in intensive breeders in Northern Italy. Samples were examined for presence of SIV by virus isolation (VI) and RT-PCR, specimens belonging to the same outbreak were pooled (max 10) for RT-PCR detection of SIV.

Virus isolation. Virus isolation (VI) was carried out by inoculation of embryonated chicken eggs (EEI) and infection of two cell culture (4): NPTr (Newborn Pig Trachea) (3) a swine origin cell line, and MDCK (Madine Darby Canine Kidney)(6) which is the most common cell culture system used for propagation of influenza virus. Two serial passages were performed. Amnio-allantoic fluids and cell supernatants, both at first and at second passage, were submitted to HA (7) and to ELISA sandwich assay (5) to evaluate the presence of influenza virus antigens.

Detection of viral RNA. Isolation of viral RNA were performed using a High Pure Viral Nucleic Acid Kit (Roche Molecular Biochemicals) according to the manufacturer instructions. cDNA was synthesized from total RNA and an aliquot was used in nested PCR reaction to amplify a conserved region of matrix gene (2). PCR products of secondary amplifications were separated using 1.5% agarose gel and visualized by staining with ethidium bromide. Sample purification, RT-PCR reactions preparations and agarose gel analysis were performed in separated laboratories. Negative controls were processed and run with each assay.

Molecular typing. Multiplex-PCR reactions to amplify haemagglutinin (HA) and neuraminidase (NA) genes were performed as previously described (1) on 56 SIVs collected in 2001-2003 years.

Serology. HI tests were performed using chicken antisera as previously described (7).

Results and discussion

Results of detection of SIV in 441 pathological samples from 177 respiratory outbreaks by diagnostic methods used in this study are shown in Table 1. RT-PCR showed the highest positivity rate (23.1%). EEI gave a percentage of positivity of 14.1, lightly lower than MDCK cell line positivity rate (15.2%), while NPTr cells showed the lowest sensitivity (11.3%). MDCK cells gave the best results in viral detection, even if this result was increased overall by using two serial passages. Considering that 46 SIVs were isolated in 177 respiratory outbreaks, it is possible to evaluate the incidence of influenza virus infection in respiratory disease complex occurred in intensive swine breeders (25.9%). RT-PCR, compared with EEI was able to detect SIV further 18 samples, even if it failed to detect the virus in two EEI positive samples. MDCK cell line allowed us to detect SIV in 7 specimens EEI negative although failed in viral isolation in 5 EEI positive cases. Furthermore inoculation of NPTr cell culture allowed SIV isolation from samples resulted EEI negative. Positivity rates of one or more methods in different combinations, among a total of 46 SIV positive specimens are showed in Figure 1. From some samples it was possible to detect SIV just by only one method when all others failed. It would be interesting to evaluate if this remark could be related to a peculiar genetic character of the isolate.

Figure 1. Detection rates of SIV in infected samples by different techniques applied alone or in combination

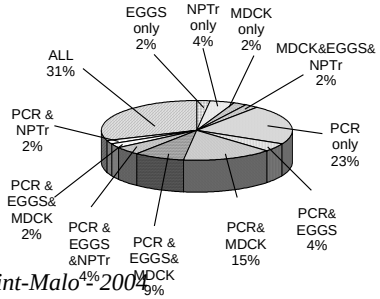

Table 1. Detection of SIV in 441 pathological samples from 177 respiratory outbreaks by various methods

	Samples examined	RT-PCR	Embryonated chicken eggs			MDCK			NPTr		
	177		I *	II	tot	I	II	tot	I	II	tot
Positive samples	46	41	20	5	25	15	12	27	13	7	20
positivity	25.9%	23.1%	14.1%			15.2%			11.3%		

*serial passage

Table 2. Results of RT-PCR subtyping of 56 SIV isolates compared to HI tests.

RT-PCR typing	N° samples	Concordant with HI	Discordant with HI	% of concordance	Sequence analysis
H1N2	25	24	1	96%	N1
H1N1	5	4	1	80%	N2
H3N2	22	22	0	100%	/
H3N2 and H1N1	4	4 (H3N2 only)	4 (H1N1 only)	/	/

Once more, adding the percentage of positivity by RT-PCR in the various combinations, it is demonstrated that this method shows the highest rate of positivity (90%). The concordance rate of the applied methods is 31%. Besides if it would be necessary to choose two combined methods for SIV detection, it is important to note that RT-PCR combined with MDCK cell line test or EEI allows to detect SIV in 94% of positive samples, while RT-PCR associated with NPTr cells inoculation reaches 96% of viral detection. The results pointed out that RT-PCR can be considered a useful tool for SIV detection in pathological samples because of its high sensitivity and short time performing. Furthermore this test could be applied to one or more pools of specimens collected in the same respiratory outbreak for a first screening before virus isolation.

At last 56 SIV isolates were RT-PCR subtyped and results were compared with HI tests (Table 2). RT-PCR typing was not in agreement with HI test in two cases: two field viruses, previously characterised as H1N2 and H1N1 by HI, were typed respectively as H1N1 and H1N2 by multiplex RT-PCR . The RT-PCR results were confirmed by sequencing. Due to the high variability of HA sequences, even if primers were designed on conserved regions obtained from multiple alignments, RT-PCR failed to amplify H1 gene in two samples. In four samples, naturally infected by two different subtype of SIV (H1N1 and H3N2), HI test detected only H3N2 subtype while RT-PCR was able to detect the presence of both subtypes. Multiplex RT-PCR showed to be useful to subtype swine influenza viruses. Results highlighted the specificity of this test to identify subtype isolates. Moreover molecular typing test resulted to be a rapid method: while for serological tests it is necessary to perform further passages to have a high HA titre viral stock, RT-PCR didn't require a high HA titre or a large amount of virus with the advantage to examine viral suspension at the first isolation. Comparison with serological tests and analysis of discordant data could be of concern for further molecular studies of particularly interesting viruses.

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