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## Viral evolution and transmission effectiveness

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### Abstract

Different viruses transmit among hosts with different degrees of efficiency. A basic reproductive number ( $R_0$ ) indicates an average number of cases getting infected from a single infected case.  $R_0$  can vary widely from a little over 1 to more than 10. Low  $R_0$  is usually found among rapidly evolving viruses that are often under a strong positive selection pressure, while high  $R_0$  is often found among viruses that are highly stable. The reason for the difference between antigenically diverse viruses with low  $R_0$ , such as influenza A virus, and antigenically stable viruses with high  $R_0$ , such as measles virus, is not clear and has been a subject of great interest. Optimization of transmissibility fitness considering intra-host dynamics and inter-host transmissibility was shown to result in strategies for tradeoff between transmissibility and diversity. The nature of transmission, targeting either a naïve children population or an adult population with partial immunity, has been proposed as a contributing factor for the difference in the strategies used by the two groups of viruses. The  $R_0$  determines the levels of threshold herd immunity. Lower  $R_0$  requires lower

herd immunity to terminate an outbreak. Therefore, it can be assumed that the outbreak saturation can be reached more readily when the  $R_0$  is low. In addition, one may assume that when the outbreak saturation is reached, herd immunity may provide a strong positive selection pressure that could possibly result in an occurrence of escape mutants. Studies of these hypotheses will give us an important insight into viral evolution. This review discusses the above hypotheses as well as some possible mechanistic explanation for the difference in transmission efficiency of viruses

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**Key words:** Basic reproductive number; Transmission; Viral infection; Antigenic diversity; Herd Immunity; Selective pressure; Influenza; Measles

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### REPRODUCTIVE RATE AND ANTIGENIC DIVERSITY

Viruses transmit between hosts with various degrees of efficiency. A parameter called basic reproductive number ( $R_0$ ) provides a quantitative estimate of the transmission efficiency.  $R_0$  is the mean number of secondary infections that one case would produce in a fully susceptible population<sup>[1]</sup>. In order for an outbreak to occur,  $R_0$  needs to be higher than 1. For some pathogens,  $R_0$  can be relatively low. For example, the 2009 pandemic H1N1 influenza virus has been estimated to have  $R_0$  of 1.3-1.7<sup>[2,3]</sup>. This

**Table 1 Basic reproductive number and antigenic diversity of certain viruses**

Virus	Reproductive No.	Antigenic diversity
Measles	12-18 <sup>[4]</sup>	Stable, single serotype, long-lasting immunity
Mumps	5-7 <sup>[4]</sup>	Stable, single serotype, long-lasting immunity
Rubella	5-7 <sup>[4]</sup>	Stable, single serotype, long-lasting immunity
Poliovirus	5-7 <sup>[4]</sup>	Stable, 3 serotypes, long-lasting immunity
Respiratory syncytial virus	1-2 <sup>[16]</sup>	Two major antigenic groups, multiple antigenic variants within the groups, re-infection can occur <sup>[17,18]</sup>
Influenza	1-2 <sup>[2,3]</sup>	Antigenic drift, numerous antigenic variants, re-infection is common

range is comparable to the  $R_0$  estimates of other pandemic and seasonal influenza viruses. It may seem surprising that a virus that can cause devastating outbreaks at a global scale has such a low transmission efficiency. Despite the low  $R_0$ , influenza virus can spread rapidly owing to its short generation time. Some other viruses have much higher  $R_0$ . For example,  $R_0$  of measles virus was estimated to be 12-18<sup>[4]</sup>. Table 1 shows  $R_0$  of some common human viral pathogens.

It is remarkable that most viruses with high  $R_0$  are antigenically stable and contain a single or a limited number of serotypes. Vaccine is usually highly effective, and escaping mutants have not been a problem. This group of virus often causes childhood diseases, such as mumps, measles, rubella, and poliomyelitis. In contrast, the antigenicity of viruses with low  $R_0$ , such as influenza virus, is highly diverse. New antigenic variants are constantly emerging to replace the preexisting strains. This so called “antigenic drift” is the most important problem in influenza vaccine production and implementation. Vaccine strains have to be predicted and selected for each outbreak season based on extensive surveillance data. Vaccination needs to be repeated annually and there is a risk of mismatch between vaccine and circulating strains<sup>[5]</sup>.

Theoretically, mutation rate and selective pressure determine the rate of evolution and diversity of any organisms. The mutation rate can explain differences between DNA and RNA viruses. Because of the lack of proof reading mechanism in the genome replication, RNA viruses have mutation rates at several orders of magnitude higher than those of DNA viruses<sup>[6]</sup>. This explains why RNA viruses are much more diverse than DNA viruses, both antigenically and phenotypically. All the above mentioned viruses with different antigenic diversity are however RNA viruses, and they were shown to have comparable high mutation rates<sup>[6]</sup>. Therefore, the determining factor for the difference in antigenic diversity must be the selective pressures. However, selective pressure is a complex phenomenon that involves several aspects of host-pathogen interaction, such as the immune response and transmission environment. While host immune responses provide positive selective pressure favoring escape mutations, constraints for optimizing transmission and replication fitness exerts negative selective pressure to keep the optimal wild type unless there are changes in transmission conditions. Viruses use different strategies to tradeoff between these two types of selective pressure resulting in different levels of diversity.

## TRANSMISSION FITNESS AS A FUNCTION OF WITHIN- AND BETWEEN-HOST DYNAMICS

Viruses transmit *via* different routes and under different conditions. In order to transmit efficiently, viruses need to shed high levels of infectious virions. However, producing high levels of progeny requires high replication rate, which makes them vulnerable to immune response or consumes too much resources (infected cells) leading to rapid progressive fatal diseases. Either way, high levels of viral replication cannot be sustained in long term. Viruses therefore need to choose either to replicate at a maximum rate in a short period, or to extend the period of replication and shedding with reduced replication rate. In order to persist in an extended period, viruses need to be able to replicate in the presence of specific immune responses and this requires an immune escape mechanism. Escape mutations leading to antigenic diversity is a common mechanism for the immune escape. A mathematical modeling showed that optimization of transmission fitness results in 3 groups of pathogens<sup>[7]</sup>: (1) childhood diseases, which are highly efficient in transmission over a short period; (2) sexually transmitted diseases, which have low transmission efficiency but can establish persistent infection; and (3) viruses with high antigenic diversity and low transmission rate, such as influenza. Contact rate may be the major determinant for the optimization options. A high contact rate, such as in childhood diseases, favors high replication and transmission rates, whereas a low contact rate favors an extended period of transmission and hence a persistent infection<sup>[8]</sup>.

## MAXIMIZING TRANSMISSION FITNESS VS TOLERATING MUTATIONS

Because viruses with high  $R_0$  are often antigenically conserved, it is plausible to assume that they are under a strong negative selective pressure. It is likely that this selective pressure stems from the necessity to maintain the optimal structure for maximum transmission and replication fitness. Because protective epitopes usually overlap with receptor-binding domains, escape mutations often affect the binding affinity to the viral receptors and impair the viral fitness<sup>[9]</sup>. The ability to tolerate mutations would likely mean that the virus has given up its optimal fitness in exchange for structural flexibility. Along this

line of evolution, a virus would optimize its fitness with a constraint to keep the flexibility. In contrast, a virus could optimize its fitness to a higher level without this constraint, resulting in a highly efficient but un-flexible structure.

## IMMUNE EFFECTIVENESS AS A BARRIER TO IMMUNE ESCAPE

A highly effective immune response provides a strong positive selective pressure that drives the emergence of an escape mutant. However, it may at the same time be a barrier for these escape mutations to occur. For example in the case of high levels of high-affinity antibodies that can recognize several minor structural changes on the virions, only drastic changes will be able to escape this antigen-antibody interaction<sup>[10]</sup>. These drastic changes will require several simultaneous mutations that may not be tolerated by the viral fitness. Similar to the case where an effective immune response targeting multiple targets, the virus will require multiple mutations to escape this response. Therefore, a highly effective immune response can be viewed as a barrier against the escape mutants.

It is well known that conserved viruses of childhood diseases elicit highly effective immune response with a lifelong protection, whereas immune response to influenza virus is short-lived and not always protective. This suggests that the effectiveness of specific immune response against those childhood diseases plays a role as a barrier to prevent the occurrence of escape mutants and antigenic diversity. On the other hand, an ineffective immune response would cause little antigenic changes on the virus because of the lack of selective pressure, and partially effective immune response may be the most effective force that drives most of the viral antigenic variation. Partially effective immune response enough to exert selective pressure, but not effective enough to suppress escape viral mutants is the most effective driving force of antigenic variation.

## HERD IMMUNITY AND SELECTIVE PRESSURE

At a population level, non-immune individuals could be protected from an infectious agent if a sufficient fraction of the population is immunized. The benefit of indirect protection is caused by the interruption of transmission chain by immunized individuals. An outbreak can be aborted if the fraction of immunized individuals reaches a threshold level. This immune protection of a partially immunized population is called herd immunity. The level of herd immunity required to terminate an outbreak depends on  $R_0$ <sup>[4]</sup>. Pathogens with higher  $R_0$  require a higher herd immunity level. The threshold herd immunity level can be calculated by  $1 - 1/R_0$ . For example, a disease with  $R_0$  of 2 will need a herd immunity level at  $1 - 1/2$  or 50% of the population, whereas a disease with  $R_0$  of

10 will need a herd immunity level of  $1 - 1/10$  or 90%. Theoretically, in a homogenous population with sufficient and evenly distributed contact rate, herd immunity level will be reached regardless of  $R_0$ . Although a virus with higher  $R_0$  requires a higher herd immunity level, it can spread more rapidly resulting in a higher number of infected cases, and the required herd immunity level will be eventually reached. In reality where populations are heterogeneous, the threshold herd immunity level may never be reached. Contact rate does not distribute evenly in a population and can vary demographically and geographically. A realistic population consists of multiple compartments interacting with one another at various contact rates<sup>[11]</sup>. Although a pathogen with a high  $R_0$  is highly effective in transmitting in a small population with a high contact rate, it may not be as effective in a larger population with multiple compartments interacting with a lower contact rate. Therefore, a high herd immunity level required to eliminate an outbreak with a high  $R_0$  may not be reached and pockets of naïve hosts will always be available to perpetuate transmission chain. In contrast, a disease with a low  $R_0$ , which requires only a low level of herd immunity, will be more likely to acquire enough herd immunity. In addition, a disease with a lower  $R_0$  depends less on high contact rate, thus it will be more effective in transmitting between compartments in a population. If an effective herd immunity level is reached, it will provide a strong selective pressure to select for immune escape. This would explain lineage extinction usually observed in influenza phylogeny and frequent emergence of a new escape variant as an antigenic drift. Without a capacity to change its antigenic epitopes, influenza would be extinct after sufficient herd immunity has been reached. Measles, on the other hand, can stay antigenically conserved because the required high herd immunity level has never been reached.

Influenza viruses are circulating in many animal species and interspecies transmission occasionally happens. Interspecies transmission can bring a new virus into human population, which would cause a pandemic due to the lack of herd immunity in the human population. However, after entering human population the course of viral evolution is mainly dictated by the interaction between the virus and human hosts since seasonal influenza viruses do not transmit back and forth between human and animal species. In contrast to the constant antigenic drift of seasonal influenza viruses, avian and swine influenza viruses do not exhibit rapid antigenic changes. Constant influx of new piglets and hatchlings as naïve hosts continues the transmission chain without the need for antigenic changes. It is interesting that the  $R_0$  in this situation does not need to be as low as those observed in human outbreaks. Although a wide range of  $R_0$  was estimated for influenza outbreaks in avian species, the number can be above 5 in some settings<sup>[12,13]</sup>. The higher  $R_0$  of avian influenza outbreaks in birds and poultry, and the antigenic stability of these viruses contrast to the lower  $R_0$  of seasonal influenza viruses with rapid antigenic

changes. This evidence supports the role of transmission effectiveness in the viral evolution strategy.

## POTENTIAL MECHANISTIC DIFFERENCES IN TRANSMISSION FITNESS

Although the difference in  $R_0$  among viruses clearly indicates that transmission fitness can vary among different viruses, mechanistic explanation for this variability is still lacking. Viral infectivity can be quantified *in vitro*, and physical quantity of viruses can be measured in genome copy number. These *in vitro* studies, however, may not accurately represent *in vivo* infectivity and transmission fitness. Theoretically, in order to obtain maximum transmission fitness, viruses should be shed in high quantity, spread effectively and stably maintained in the environment. They should also be able to efficiently penetrate protective barriers to the target cells after entering a new host, bind to target cells with high affinity, withstand host extra- and intra-cellular innate defense. Among these contributing factors, receptor-binding affinity is probably the most crucial one since most neutralizing epitopes overlap with receptor-binding domains on viral surface proteins. Optimization of the receptor binding may exert structural constraints on mutations of the antigenic epitopes. Evidence for any of these possible differences is still lacking. For example, viral load in respiratory secretion as quantified in viral RNA copy number did not seem to be different between influenza and measles<sup>[14,15]</sup>, although the data were from different studies and the comparison may not be fully reliable. Similarly, direct comparison of the other viral characteristics between viruses with high and low  $R_0$  should be studied. This will improve our understanding of these viruses.

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## Emergence of Toscana virus in the mediterranean area

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### Abstract

Toscana virus (TOSV) is an arthropod-borne virus, identified in 1971, from *Phlebotomus perniciosus* and *Phlebotomus perfiliewi* in central Italy. TOSV belongs to the *Phlebovirus* genus within the *Bunyaviridae* family. As other bunyaviruses, the genome of TOSV consists of 3 segments (S for small, M for Medium, and L for Large) respectively encoding non structural and capsid proteins, envelope structural proteins, and the viral RNA-dependant RNA-polymerase. It is transmitted by sand flies. Therefore its distribution is dictated by that of the arthropod vectors, and virus circulation peaks during summertime when sandfly populations are active. Here, we reviewed the epidemiology of TOSV in the old world. First evidence of its pathogenicity for humans, specifically its propensity to cause central nervous system (CNS) infections such as meningitis and encephalitis, was reported in central Italy. After 2000, it was recognized that TOSV had a much larger geographic distribution than initially believed, and was present in most of the Western European countries

located on the northern border of the Mediterranean Sea (Portugal, Spain, France, Greece, Croatia) as well as eastern countries such as Cyprus and Turkey. In the countries where TOSV is present, it is among the three most prevalent viruses in meningitis during the warm seasons, together with enteroviruses and herpesviruses. Up to now, epidemiological data concerning Northern Africa and other countries located south of the Mediterranean are scarce. TOSV must be considered an emerging pathogen. Despite the important role played by TOSV in CNS infections, it remains a neglected agent and is rarely considered by physicians in diagnostic algorithms of CNS infections and febrile illness during the warm season, probably because of the lack of information.

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**Key words:** Toscana virus; Phlebovirus; Emergence; Europe; Meningitis; Fever; Sandfly; Phlebotomus; Zoonosis; Arthropod-borne

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### HISTORY OF TOSCANA VIRUS

In 1971, a previously unrecognized phlebovirus, Toscana virus (TOSV), was isolated in central Italy from two different species of sand flies, *Phlebotomus perniciosus* (*P. perniciosus*) and *Phlebotomus perfiliewi* (*P. perfiliewi*)<sup>[1-3]</sup>. It was only 15 years later that the first cases of TOSV infection were reported from returning travelers who had visited the

region where TOSV was initially described<sup>[4]</sup>. Additional evidence that TOSV was frequently infecting humans in regions where sandflies were present and that TOSV was a major cause of central nervous system (CNS) infections during the warm season were published first in Italy, then in other Mediterranean countries (Spain, France, Cyprus, Portugal, Greece, Turkey, Tunisia). A bibliographic search using “Toscana virus” as keyword in the PubMed database retrieved 166 research and review articles. Despite increasing evidence of its major role in human diseases, there is little awareness of physicians concerning the public health impact of TOSV which merits to be considered from April to October as a possible cause of febrile illness with or without CNS involvement in the Mediterranean area.

TOSV is an arthropod-borne virus with a negative-sense, single-stranded RNA genome consisting of 3 segments as other members of the genus *Phlebovirus* within the family *Bunyaviridae*. TOSV is a serotype of the species *Sandfly fever Naples virus* that currently includes also Sabin, Tehran and Karimabad viruses. Recently, novel phleboviruses (Massilia virus, Granada virus, Punique virus) closely related to but clearly distinct from TOSV, have been discovered and are likely to belong to this species<sup>[5]</sup>, albeit yet not classified by the International Committee for Taxonomy of Viruses.

## NATURAL CYCLE

### Vectors

TOSV was isolated from *P. perniciosus* and *P. perfiliewi* but never from *P. papatasi*<sup>[1-3]</sup>. *P. perniciosus* is distributed throughout the Mediterranean region in two races. The most probable transmission vector for TOSV in Spain is *P. perniciosus* since about 70% of captured individuals corresponded to this species. TOSV was detected both in male and in female pools of phlebotomine sand flies, in Italy, Spain and France, at comparable rates. The detection of virus in pools of males flies suggests vertical and/or sexual transmission of TOSV among phlebotomine sand flies. Transovarial transmission of TOSV in sand flies has been demonstrated in laboratory experiment and by virus isolation from male phlebotomine flies. Venereal transmission from infected male to uninfected female has also been demonstrated. However, the respective importance of the different routes of transmission of TOSV in sand flies is not clearly elucidated.

### Reservoir of TOSV

Neither wild mammals nor birds were recognized as reservoir, but few studies were carried to have a clear idea whether an animal reservoir host exists for TOSV or not<sup>[3]</sup>. Several phleboviruses have been isolated from the blood of sick persons and from wild animals. However, the importance of vertebrates in the maintenance of cycle of these agents remains unclear owing that viremia is low and transient in susceptible laboratory animals, and that large quantity of virus must be ingested in order to infect experimentally the sand flies<sup>[6-9]</sup>. Whether human

can play a role in the virus cycle by infecting naïve sand flies is unknown, but this mechanisms is generally believed to play a negligible role at best. The present dogma is that competent species of sand flies might be the reservoir of TOSV. However, the array of evidence is low and additional studies are needed to confirm or contradict this hypothesis.

**Disease in humans and TOSV:** In the scientific literature, the contrasting unbalance between the number of acute infections (case reports and series) and seroprevalence data suggests that a significant proportion of TOSV infections are either non symptomatic or causes mild symptoms that do not justify to visit a general practitioner or an infectious diseases specialist; this situation precludes laboratory confirmation by specific virological assays, and leads to underestimate drastically the number of non neuro-invasive forms of TOSV human infection. Because of the recent discovery of phleboviruses that are distinct from but genetically and antigenically related to TOSV, seroprevalence figures must be interpreted carefully because of the possible cross-reactivity between those viruses and TOSV<sup>[10,11]</sup>. Owing this situation, the most documented clinical form of TOSV infection consists of neuro-invasive cases which are generally hospitalized and therefore can benefit from laboratory documentation assessing the etiologic role of TOSV. The incubation is usually short (3-7 d with a maximum of 2 wk) and is likely influenced by the virus load of the inoculum. The onset is brutal with headache (100%), fever (76%-97%), nausea and vomiting (67%-88%), and myalgias (18%). Physical examination shows a neck rigidity (53%-95%), Kernig sign (87%), consciousness troubles (12%), tremors (2.6%), paresis (1.7%), nystagmus (5.2%). Cerebrospinal fluid (CSF) usually contains more than 5-10 cells with normal levels of glucose and proteins. In blood, leucocytosis (29%) or leucopenia (6%) can be observed<sup>[12]</sup>. The mean duration of the disease is 7 d and the outcome is usually favorable. It is impossible to distinguish aseptic meningitis due to TOSV from meningitis due to other pathogens on the basis of clinical manifestations. Few severe cases have been reported in the literature: they consist of pure encephalitis or meningo-encephalitis<sup>[13-17]</sup>. Scarce information is available on other clinical forms, which consist mainly in peripheral neurological disorders mostly reported as single case reports<sup>[18]</sup>. To date, there is no data published suggesting that TOSV could cause manifestations other than those aforementioned. However, there is to our knowledge no study that was designed to investigate TOSV potential to cause other manifestations in humans. Regarding the monthly distribution of human cases of TOSV infections, all studies are congruent: the higher risk of acquiring TOSV is in August (++++), then July and September (++), and finally June and October (+)<sup>[12]</sup>.

## GEOGRAPHIC DISTRIBUTION OF TOSV

### Italy

TOSV was isolated in patients presenting with menin-

gitis after returning from Italy<sup>[4,19]</sup>. A pioneer retrospective study (1977-1988) demonstrated that TOSV was a prominent cause of summer meningitis in the Tuscany and Marche regions<sup>[20,21]</sup>. In Central Italy, TOSV accounted for the first cause of CNS infections during the warm season far ahead other viruses with incidence ranging 30%-52%<sup>[22,23]</sup> in adult and children. TOSV was also isolated in other regions of Italy such as Emilia Romagna<sup>[24,25]</sup>, Piedmont<sup>[24]</sup>, Sardinia<sup>[26,27]</sup>, Sicilia<sup>[28]</sup>, and Umbria<sup>[24]</sup>.

### France

The first case of TOSV infection acquired in France was reported for a German traveller returning from Southern France<sup>[29]</sup>. During the National surveillance of West Nile virus in Southern France, samples obtained in the Public Hospital systems of Marseille, the second largest French city (800 000 inhabitants) were also tested for TOSV first sporadically and then systematically from 2007: three cases of meningitis and one isolated fever<sup>[30,31]</sup>, one encephalitis<sup>[17]</sup> were reported. In the 2 seroprevalence studies conducted with volunteer blood donors living in southeastern France (including Corsica Island)<sup>[32,33]</sup>, rates ranging from 6.5% to 19% with average values at 12% were observed. In south-eastern France, TOSV is among the three most prominent causes of aseptic meningitis during the warm season together with enteroviruses and herpesviruses (herpes simplex virus and varicella-zoster virus).

### Spain

The first case of TOSV infection reported from Spain occurred in a Swedish tourist after visiting Catalonia and was documented by means of neutralization assay<sup>[34]</sup>. During the last decade, many cases of TOSV infection were reported in Spain, and several comprehensive epidemiological studies established that TOSV was one the three main cause of meningitis and a prominent agent of human infections with seroprevalence rates ranging 5%-26.2% in several regions of Spain such as Madrid, Granada, and the Mediterranean coast<sup>[35-38]</sup>. These data suggest that the situation in Spain is similar to that observed in France with lower prevalence in CNS infections compared to what was observed in central Italy.

### Cyprus

Several studies were conducted in Swedish United Nations soldiers based in Cyprus in 1985. One case of seroconversion was documented in a total of 298 soldiers stationed in Cyprus during 6 mo without any clinical manifestation<sup>[39]</sup>. Seroprevalence studies showed that 20% (96/479) of the healthy population of the island had TOSV IgG<sup>[40]</sup>.

### Greece

Seroprevalence studies have recently detected high rates of IgG against TOSV in populations living in two Greek islands in the Ionian Sea, Corfu (51.7%) and Cephalonia

(39%). Up to now there are no studies on meningitis or encephalitis cases caused by TOSV in Greece. To date, the only evidence about TOSV infection in Greece was reported in 1997 in a 73-year-old patient who stayed 3 wk in the region of Athens and presented with meningitis and was documented by the presence of IgM and IgG through immunofluorescence test<sup>[29]</sup>. So far there is no absolute confirmation based on virus isolation or reverse-transcription polymerase chain reaction (RT-PCR) detection of TOSV.

### Portugal

It is in a patient returning from Portugal that the first human strain of TOSV was isolated, thus demonstrating the potential of this virus to infect humans and to cause neuro-invasive symptoms<sup>[41]</sup>. The second case to be reported is traced back in 1996<sup>[42]</sup>. From 2002 to 2005, 106 CSF samples, collected between June and September in patients younger than 30-year-old, were tested for TOSV by RT-PCR, and resulted in the detection of 6 positives<sup>[43]</sup>. In a seroprevalence study targeting 538 patients who were suspect of vector-borne virus infections between 2004 and 2008, 4.2% of those with neurological signs, and 1.3% of those without neurological signs were found to contain IgG reactive against TOSV<sup>[44]</sup>.

### Germany

Seroepidemiological surveys conducted showed than at the end of the 1990's, TOSV was not present in Germany and that the cases were imported from endemic countries (Italy, Portugal, France)<sup>[45]</sup>. Given the fact that sand flies are occupying a expanding geographic area, possibly due to climatic changes, it is now desirable to conduct studies on TOSV in regions of Europe located north of the historical limit of sandfly circulation.

### North Africa (Tunisia, Algeria and Morocco)

An increasing number of studies suggest that TOSV might be present in the countries located on the southern border of the Mediterranean. However, so far there is no undisputable evidence since TOSV has not been isolated either from sand flies or from human specimens. Although the presence of TOSV in this region is expected, the present data consist of serological tests<sup>[46]</sup> which are prone to cross-reaction that may confuse between TOSV and genetically-related phleboviruses that have been recently discovered or detected (Punique virus, Algeria virus)<sup>[47,48]</sup>. Therefore clinical and entomological studies are necessary to clarify this point.

### Kosovo

To date, there is only one seroprevalence study performed in Kosovo<sup>[49]</sup>. A total of 11 out of 200 sera (5.5%) were found to be positive by immunofluorescence and enzyme linked immunosorbent assay (ELISA); plaque reduction neutralization test (PRNT) confirmation indicated that one sera contained antibodies specific for TOSV.

**Table 1** Reverse transcriptase polymerase chain reaction systems described in the literature for Toscana virus

Name	Sequence	Gene	Assay	Ref.
TV1	CCAGAGGCCATGATGAAGAAGAT	N	RT-PCR	[56]
TV2	CCACTCCTATGAGCAGCTTCT	N	RT-PCR	
TV3	AACCTGATTTCAGTCTACCAGTT	N	Nested	
TV4	TTGTTCTCAGAGATGGATTATG	N	Nested	
TosN123	GAGTTTGCTTACCAAGGGTTTG	N	RT-PCR	[37]
TosN829	AATCCTAATCCCCTAACCCCC	N	RT-PCR	
TosN234	AACCTTGTGAGGGGNAACAAGCC	N	Nested	
TosN794	GCCAACCTTGGCGGATACTTC	N	Nested	
NPhlebo1+	ATGGARGGTTTGTIWSICIIC	L	RT-PCR	[37]
Nphlebo1-	AARTTRCTIGWIGCYTTIARIGTIGC	L	RT-PCR	
Nphlebo2+	WTICCIAICCIYMSAARATG	L	Nested	
Nphlebo2-	TCYTCYTTRTTYTRARRTARCC	L	Nested	
ATos2-	RTGRAGCTGGAACKGGIGWIG	L	Nested	[58]
TosS1+	CAGAGATTCCCGTGTATTAAC	N	Nested	
TosS1-	GAGTGCTGCCAAGTCTTATGAC	N	Nested	
TosS2+	CAGAGATTCCCGTGTATTAACAAAAGC	N	Nested	
TosS2-	TAGAGAACTGCTCTTCCACC	N	Nested	[56]
T1	CTATCAACATGTCAGACGAG	N	RT-PCR	
T2	CGTGCTCTGTCAGAATCCCT	N	RT-PCR	
T3	CATTGTTCAAGTGGTCAA	N	Nested	
T4	CGTGCTCTGTCAGAATCCCT	N	Nested	[65]
Phlebo F1	TTTGCTTATCAAGGATTGATGC	N	RT-PCR	
Phlebo F2	TTTGCTTATCAAGGATTGACC	N	RT-PCR	
Phlebo rev	TCAATCAGTCCAGCAAAGCTGGGATGCATCAT	N	RT-PCR	
SFNV-S1	CTTYTTRTCYCTCTRGTAAGAA	N	RT-PCR	[64]
SFNV-R1	ATGATGAAGAARATGTGAGAGAA	N	RT-PCR	
SFNV-S2	GCRGCCATRTTKGGYTTTCAAA	N	Nested	
SFNV-R2	CCTGGCAGRGACACYATCAC	N	Nested	
STOS-50F	TGCTTTCTTGATGAGTCTGCAG		rt RT-PCR	[59]
STOS-138R	CAATGCGCTTYGGRTCAA		rt RT-PCR	
STOS-84T-FAM	ATCAATGCATGGGTRAATGAGTTTGCTTACC		rt RT-PCR	
TOS FP	GGGTGCATCATGGCTCTT		rt RT-PCR	
TOS P	CAATGGCATCCATAGTGGTCCCAGA		rt RT-PCR	[60]
TOS RP	GCAGRGACACCATCACTCTGTC		rt RT-PCR	

### Elba

Two case reports demonstrate that TOSV is present on Elba Island<sup>[50,51]</sup>. However, there is no data on the seroprevalence in the island.

### Turkey

In contrast in Turkey, there is indisputable evidence that TOSV is present and causes human infections<sup>[52,53]</sup>. Sera of blood donors from the Ankara, Konya, Eskisehir and Zonguldak provinces of Turkey were screened by indirect immunofluorescence test IIFT and confirmed by virus neutralization: neutralising antibodies were found in sera from the 4 provinces<sup>[53]</sup>. Today, 21 provinces of Turkey are known to have TOSV circulation<sup>[53]</sup>. In addition, 16 out of 102 patients presenting with CNS infections of unknown aetiology were positive by real-time RT-PCR for TOSV, which was subsequently isolated and demonstrated to belong to the genotype A together with sequences derived from Italian and French strain<sup>[53]</sup>.

### Mediterranean islands situation

It merits to be analysed owing the specific ecological conditions. As a matter of facts, TOSV has been proved to be present in many Mediterranean islands (Elba, Cyprus, Sardinia), or is suspected to circulate based on serological data such as Majorca<sup>[54]</sup>, and Corsica<sup>[31]</sup>.

### Detection of TOSV in human and animal samples and in sandfly specimens:

Seroconversion and detection of IgG and/or IgM can be achieved using a large variety of techniques such as complement fixation, hemagglutination inhibition, ELISA. ELISA tests have been developed with either crude antigens or purified virus obtained from infected cells. The advantage of ELISA resides in its capacity to tests rapidly a large number of specimens. An ELISA test based on recombinant nucleoprotein gene was developed and is now commercialized. Many seroprevalence studies using this commercial test were recently published<sup>[31,35,38]</sup>.

However, cross-reactivity exists between phleboviruses, and confounding results have to be expected. This is particularly critical between viruses that are antigenically related to TOSV such as the other members of the *Sandfly fever Naples virus* species (Tehran, Naples, Sabin) and the viruses for which close relationships have been demonstrated (Massilia, Punique, Granada)<sup>[10,11,48]</sup>. Accordingly, the presence of antibodies reacting with TOSV antigens must not be interpreted as an undisputable evidence of infection with TOSV, but rather a sign of infection with a phlebovirus that is antigenically related with TOSV, but might be drastically distinct from TOSV. The recent discovery of novel phleboviruses should engage to revisit the conclusions of these studies and to be careful in the interpretation of such results in the future.

PRNT is the test of choice when definitive confirmation of the virus identity, either at the species level or even at lower level, is necessary. In theory, PRNT should be used to confirm all results provided in seroprevalence studies by techniques other than PRNT. However, this approach remains difficult and time consuming since it demands to possess the strains of the different phleboviruses and to standardize the assay.

Direct diagnosis can rely on virus isolation or molecular detection of the viral genome. Isolation of the virus from clinical samples can be achieved by using CSF at the acute stage of the infection. Virus isolation and molecular detection is also possible from blood as recently assessed<sup>[53]</sup>. TOSV replicates in Vero, BHK-21, CV-1, SW13 cells with cytopathic effect and not in C6/36 cells<sup>[2,55]</sup>. The most efficient technique remains to inoculate the biological material into the brain of newborn mice, but owing it requires appropriate facility and legal agreement it is performed in specialized centers only. Although it is known that virus isolation has a lower sensitivity compared to RT-PCR, the limited number of TOSV isolates justifies to attempt isolation whenever it is possible for a better understanding of the genetic and antigenic diversity of TOSV.

Different methods for molecular diagnosis of TOSV have been developed and published (Table 1). Up to 2005, all studies were performed with classic PCR detection based on single round or nested protocols<sup>[21,37,56,57]</sup>. The description of two genotypes of TOSV supports the need of special attention when designing primers and probes in order to avoid false negative results<sup>[58]</sup>. Recently, real-time RT-PCR techniques have demonstrated a huge improvement: (1) by reducing the time to obtain the result; (2) by improving the sensitivity; and (3) by reducing the risk of cross-contamination<sup>[59,60]</sup>. In addition to TOSV RNA detection in CSF, TOSV RNA was recently detected by real time RT-PCR in the serum of 16 patients presenting with CNS symptoms<sup>[53]</sup>. Few strains of TOSV are currently genetically characterized. It is therefore pivotal to pursue this effort in order to better understand the genetic diversity that exist within TOSV isolated and between TOSV and other closely-related phleboviruses in the species *Sandfly fever Naples virus*, in order to adapt the diagnostic system for a improved detection.

**Genetic diversity of TOSV strains:** Phylogenetic analysis has demonstrated that TOSV isolates from Spain differ from that originating from Italy<sup>[58]</sup>. Based on the analysis of the GN glycoprotein (M RNA segment), 4 lineages of TOSV have been proposed<sup>[61]</sup>. Within TOSV sequences obtained from Italy, minor differences (no more than one amino acid substitution) were observed the nucleocapsid gene of strains isolated from 1980 to 1998 from *P. perniciosus*, *P. perfiliewi*, and humans<sup>[62,63]</sup>. Eleven Spanish strains also showed a very low genetic diversity within the polymerase gene<sup>[37]</sup>. In France, TOSV of both genotypes have been reported not only in sand flies but also in patients with meningitis: it seems that

there is no difference in the clinical picture or diseases severity that might be linked to genotype specificity<sup>[64]</sup>.

## CONCLUSION

Despite undisputable evidence that the geographic area where TOSV is circulating is larger than initially believed, TOSV remains a neglected human pathogen that will merit massive investigations for a better understanding. To achieve this objective, there is a need for transdisciplinary studies addressing various aspects such as (1) the epidemiology of the infection in human and animal populations; (2) the ecological factors impacting on the spread of phlebotomine vectors and relationships between TOSV and Leishmania parasites; (3) the influence of other phleboviruses co-circulating with TOSV in the epidemiology and transmission; and (4) the necessity to decipher the transmission routes of TOSV within sand flies through field studies in nature and experimental studies taking advantage of insectarium facilities.

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## Infectious laryngotracheitis virus in chickens

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### Abstract

Infectious laryngotracheitis (ILT) is an important respiratory disease of chickens and annually causes significant economic losses in the poultry industry worldwide. ILT virus (ILTV) belongs to alphaherpesvirinae and the *Gallid herpesvirus 1* species. The transmission of ILTV is *via* respiratory and ocular routes. Clinical and post-mortem signs of ILT can be separated into two forms according to its virulence. The characteristic of the severe form is bloody mucus in the trachea with high mortality. The mild form causes nasal discharge, conjunctivitis, and reduced weight gain and egg production. Conventional polymerase chain reaction (PCR), nested PCR, real-time PCR, and loop-mediated isothermal amplification were developed to detect ILTV samples from natural or experimentally infected birds. The PCR combined with restriction fragment length polymorphism (RFLP) can separate ILTVs into several genetic groups. These groups can separate vaccine from wild type field viruses. Vaccination is a common method to prevent ILT. However, field isolates and vaccine viruses can establish latent infected carriers. According to PCR-RFLP results, virulent field ILTVs can be derived from modified-live vaccines. Therefore, modified-live vaccine reversion provides a source for ILT outbreaks on chicken farms. Two recently licensed commercial recombinant ILT vaccines are also in use. Other recombinant and gene-deficient vaccine candidates are in the

developmental stages. They offer additional hope for the control of this disease. However, in ILT endemic regions, improved biosecurity and management practices are critical for improved ILT control.

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**Key words:** Infectious laryngotracheitis virus; Infectious laryngotracheitis; *Gallid herpesvirus-1*; Polymerase chain reaction combined with restriction fragment length polymorphism; Recombinant laryngotracheitis vaccines

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### GENERAL BIOLOGY OF INFECTIOUS LARYNGOTRACHEITIS VIRUS

Infectious laryngotracheitis (ILT) is a member of the Herpesviridae, the genus *Iltovirus* and *Gallid herpesvirus 1* species<sup>[1-3]</sup>. It was first reported in the 1920s; however, this disease may have existed in chickens much earlier<sup>[4]</sup>. The genome of ILT virus (ILTV) is linear and approximated 150 kb of double-stranded DNA. It consists of long and short unique regions (UL, US) and two inverted repeat sequences (internal repeat; terminal repeat) that flank the US regions. An assembled complete genome sequence of ILTV from different strains contains 148 665 base pairs, and a G + C content of 48.16%. It was predicted that the genome had 77 open reading frames (ORFs), and 63 of these were homologous to herpes simplex virus-1 genes<sup>[1-5]</sup>. Most recently, 2 research groups used robust high-throughput methods to sequence the full length of vaccine and virulent ILTVs. The Australian group se-

quenced the complete genome of 3 chicken-embryo origin (CEO) vaccines, which were European strain, Serva, and 2 Australian strains, SA2 and A20. The genomes of the three CEO vaccines had 99% identity, and few single nucleotide polymorphisms were identified. However, the virulence was different between SA2 and A20 ILTVs<sup>[6,7]</sup>. An American research group sequenced the complete sequences of 4 virulent strains from different genotypes, and compared them with the Serva strain and the composite sequences. The vaccine and virulent ILTVs were 150, 335 to 153, 633 base pairs with 80 predicted ORFs. They are highly conserved and only 4 ORFs were different in length and 13 to 49 amino acids changed<sup>[8]</sup>. These full genomic sequences contributed to further knowledge of the viral pathogenic factors, gene functions, and vaccine development. Although ILTV, Marek's disease virus, and herpesvirus of turkey (HVT) belong to Alphaherpesvirinae, neither the nucleotide sequence nor the deduced amino acid sequence of glycoprotein D and ICP27 were similar. According to phylogenetic analysis, ILTV could be an early type of  $\alpha$ -herpesviruses<sup>[9,10]</sup>.

ILTV is an enveloped virus and sensitive to heat, ether, chloroform, and other lipolytic solvents<sup>[11]</sup>. Different strains of ILTV have different resistance to heat. At lower temperatures, ILTV maintains infectivity for a long period. The virus survived for days to months at 13–23 °C in tracheal exudates and chicken carcasses<sup>[12]</sup>. When stored at -20 °C to -60 °C, ILTV was viable for months to years. Storage media containing glycerol or sterile skim milk greatly increases the infectivity in tracheal swabs<sup>[13,14]</sup>. It has been shown that the virus was destroyed in 1 min by treated with 3% cresol or a 1% lye solution<sup>[11]</sup>. On a chicken farm, 5% hydrogen peroxide mist administered with fumigation equipment completely inactivated ILTV<sup>[15]</sup>.

### Hosts of ILTV

All ages of chickens are affected, but chickens older than 3 wk are most susceptible to ILTV<sup>[16]</sup>. It has been shown that ILTV can infect pheasants, pheasant-bantam crosses, and peafowl<sup>[17]</sup>. ILTV can infect turkeys at about 100 d of age. Clinical signs of dyspnea and depression can be observed in infected turkeys<sup>[18]</sup>. Other avian species are resistant to ILTV infection<sup>[17,19,20]</sup>. Embryonating chicken eggs are the most common method for propagating ILTVs. In chicken embryos, ILTV forms plaques on the chorioallantoic membrane (CAM). The plaques can be observed 48 h after infection, and embryos can die in 2–12 d post infection (PI). Strains of ILTV showed different plaque size and morphology on the CAM<sup>[21–24]</sup>. The ILTV can be isolated in primary cell cultures, such as chicken embryo liver (CEL), chicken embryo kidney (CEK), and chicken kidney (CK) cell cultures. The sensitivity of ILTV isolation and propagation from field samples vary depending on the type of cell cultures. CEL was the most sensitive for isolation, followed by CK. The CEK and chicken embryo lung cells were less sensitive. Chicken embryo fibroblasts, Vero cells, and quail cells were not satisfactory

**Table 1** Viral isolation from swabs in specific-pathogen-free embryonating eggs after sanitizer treatments

Sanitizer	Sample	1st d	7th d	14th d	21st d
Sodium hypochlorite	Trachea	- <sup>1</sup>	-	+ <sup>2</sup>	+
	Drinker	-	-	+	+
	Biofilm	ND	ND	ND	+
Citric acid	Trachea	-	-	+	+
	Drinker	-	+	+	+
	Biofilm	ND	ND	ND	+
Sodium hydrogen sulfate	Trachea	-	-	-	-
	Drinker	-	-	-	-
	Biofilm	-	-	-	-
Hydrogen peroxide	Trachea	-	-	-	-
	Drinker	-	-	-	-
	Biofilm	-	-	-	-
Positive control <sup>3</sup>	Trachea	-	-	+	+
	Drinker	-	+	+	+
	Biofilm	+	+	+	+
Negative control <sup>4</sup>	Trachea	-	-	-	-
	Drinker	-	-	-	-
	Biofilm	-	-	-	-

<sup>1</sup>Negative for virus isolation; <sup>2</sup>Positive for virus isolation; <sup>3</sup>Water lines received vaccine, but no sanitizer; <sup>4</sup>Water lines received no vaccine or sanitizer. ND: Not done.

**Table 2** Infectious laryngotracheitis virus isolation from beetles in specific-pathogen-free embryos

	Days after outbreak	Before H <sub>2</sub> O <sub>2</sub> treated	After H <sub>2</sub> O <sub>2</sub> treated
Farm 1	17	-1	-
	53	-	-
	103	-	-
Farm 2	13	2	+
	42	+	-
	90	-	-

Farm 1: Negative for virus isolation; Farm 2: Positive for virus isolation.

for primary isolation of ILTV. Lymphocytes, thymocytes, and activated T cells were not sensitive to ILTV infection<sup>[24–26]</sup>.

**Transmission and latent infection:** Natural transmission of ILTV is through the upper respiratory and ocular routes. Sources of ILTV are clinically affected chickens, latent infected carriers, contaminated dust, litter, beetles, drinking water and fomites<sup>[13,27,28]</sup>. Our recent study showed that ILTV can remain in biofilm of drinking water lines and subsequently be transmitted to susceptible birds. Biofilm is a sticky substance produced by bacteria, which can render microorganism resistant to some routinely used sanitizers. Commercial sodium hydrogen sulfate (pH water treatment, PWT<sup>®</sup>, Jones-Hamilton Co., Walbridge, OH) and hydrogen peroxide (Proxy-Clean<sup>®</sup>, Kanters Special Products USA, St. Paul, MN) were able to inactivate ILTV in the water lines<sup>[27]</sup> (Table 1). Darkling beetles in the chicken farms are possible sources to transmit ILTV. Our investigation revealed that in ILTV infected chicken houses, the darkling beetles contained live virus at least 42

d after the disease outbreak<sup>[28]</sup> (Table 2). Other possible sources of transmission included dog, crows, and cats<sup>[29]</sup>. Wind-borne transmission between farms was critical for ILTV spread<sup>[30]</sup>. The tissue culture origin (TCO) and CEO vaccines served as a model for ILTV transmission as well as replication *via* eye drop route. The viruses replicated mainly in the conjunctiva and trachea. The vaccine viruses can be re-isolated and viral DNA can be detected from contact exposed birds as early as 7 d after exposure<sup>[31]</sup>.

ILTV can persist in the infected birds. The virus can be re-isolated from tracheal swabs 7 wk PI, or 2 mo PI in tracheal samples. The trigeminal ganglion is the target for ILTV latency. Fifteen months after vaccination, ILTV in the trigeminal ganglion was reactivated. In mature laying chickens challenged with virulent ILTV, DNA was detected in the trigeminal ganglion by polymerase chain reaction (PCR). The ILTV DNA can be detected in the trigeminal ganglion at 2 d after birds were vaccinated *via* eye drop route. When birds were stressed, such as the onset of lay or re-housing, ILTV was re-activated and spread to susceptible birds<sup>[31-33]</sup>.

**Clinical signs and lesions:** There are two clinical forms of ILT infection (severe and mild). Clinical signs of the severe form include dyspnea and bloody mucus. This form can cause high morbidity and mortality up to 70%<sup>[13,34]</sup>. The mild form includes depression, reduced egg production and weight gain, conjunctivitis, swelling of the infraorbital sinuses (almond shaped eyes), and nasal discharge. ILT takes 10 to 14 d for recovery, but with some strains the clinical signs may extend for weeks<sup>[35]</sup>. The mild form is the most commonly seen type in the US and is called “silent, vaccinal, or almond-shape eye” ILT.

Gross lesions are observed in the larynx and trachea. With the severe form, the mucosa of the respiratory tract shows inflammation and necrosis with hemorrhage. A characteristic feature is intranuclear inclusion bodies in epithelial cells. Inclusion bodies are generally present for a few days at the early stage of infection before epithelial cells die. Epithelial cells also form multinucleated cells (syncytia). When the necrotic epithelial cells detached from the trachea, bloody mucus was observed<sup>[35,36]</sup>.

### Detection and identification of ILTV

Laboratory diagnosis is required for ILT, because other diseases cause similar clinical signs and lesions, such as infectious bronchitis, Newcastle disease, avian influenza, infectious coryza, and mycoplasmosis. ILTV infection can be confirmed using several methods, including virus isolation and DNA detection. A commercial ELISA for detection of antibodies against ILTV is available; however, the test is not used for routine laboratory diagnosis. For ILTV isolation, the CAM inoculation of 9-to-12-d-old embryos and primary cell culture are used. Swab or organ samples from the trachea, conjunctiva, larynx, and lung of clinically affected birds are collected and inoculated on

the CAM. The CEL and CK cell cultures were suitable for ILTV isolation. Multinucleated giant cells may be observed 24 h PI<sup>[24,35]</sup>.

Traditional antigen detection uses ILTV polyclonal or monoclonal antibodies to bind ILTV antigen from clinical samples. Viral antigen was detected using direct or indirect fluorescent antibodies (FA) in the tracheal smear or tracheal tissues<sup>[37]</sup>. A more sensitive method using immunoperoxidase (IP) labeled monoclonal antibodies can be used as immunoprobe to detect ILTV in tracheal smears. This IP method detected ILTV on the second day PI<sup>[38]</sup>. Agar gel immunodiffusion uses hyperimmune serum against ILTV to detect antigen in tracheal samples and it can differentiate ILT from the diphtheritic form of fowl pox. However, the sensitivity was lower than with other methods<sup>[39]</sup>. Antigen capture enzyme-linked immunosorbent assay (AC-ELISA) uses ILTV monoclonal antibodies for antigen detection. The AC-ELISA was faster and more accurate than AGIP or FA<sup>[40]</sup>.

ILT DNA detection methods have developed rapidly in recent years. These methods can identify ILTV quickly, accurately, and are highly sensitive. Molecular techniques for ILTV detection include cloned DNA probes for dot-blot hybridization, PCR, nested PCR, real-time PCR, multiplex PCR, *in situ* hybridization<sup>[41-49]</sup>, and PCR followed by restriction fragment length polymorphism (RFLP)<sup>[43,50-52]</sup>. ILTV detection with PCR was more sensitive than virus isolation in cell culture and electron microscopy. PCR also detected ILTV in the samples, which were contaminated with other pathogens<sup>[53]</sup>. Compared with electron microscopy, histologic test, viral isolation *via* CAM route, FA test, and real-time PCR for ILT diagnosis in a broiler farm outbreak, the real-time PCR was the most sensitive method for ILTV detection<sup>[54]</sup>. Since many laboratories are not set up for real-time PCR; FA, histopathology, and PCR are the most commonly used methods used for routine diagnosis of ILT<sup>[54]</sup>.

Recently, we developed a novel nucleic acid detection method, loop-mediated isothermal amplification (LAMP), for ILTV DNA identification and compared the sensitivity and specificity with real-time PCR. Both methods are highly specific and sensitive. The LAMP assay can detect ILTV DNA at the concentration of 60 copies/ $\mu$ L in 45 min without expensive equipment and reagents. The real-time PCR has a detection limit at 10 copies/ $\mu$ L<sup>[55]</sup>. The LAMP assay is suitable for basic diagnostic laboratory detection in the field and real-time PCR can be used for further verification.

### ILTV strain differentiation

It is not possible to identify different strains of ILTV by serological methods, because ILTVs have close immunodominant domains<sup>[56]</sup>. The most common and effective molecular method for ILTV differentiation is PCR followed by RFLP. PCR-RFLP analysis of single or multiple viral genome regions can differentiate strains from various geographic areas and vaccine from field strains<sup>[43,57-60]</sup>. Restriction endonuclease analysis of ILTV DNA can

differentiate vaccine strains from wild type strains<sup>[61]</sup>. Moreover, PCR-RFLP analysis of the partial ICP4 gene, gC gene, and TK gene can distinguish field strains from vaccines. However, some virulent isolates could not be separated from vaccine strains<sup>[43]</sup>. Han *et al.*<sup>[62]</sup> (2001) analyzed multiple genes with PCR-RFLP combined with DNA sequence analysis of the gG and TK genes to differentiate vaccine and non-vaccine strains. Researchers demonstrated that multiple gene PCR-RFLP was more reliable to differentiate vaccines from field strains<sup>[50]</sup>. A new reverse RFLP method was reported to separate vaccine from non-vaccine ILTV strains. This method combined real-time quantitative PCR and restriction enzyme digestion and calculated the change of cycle threshold number value between digested and undigested template DNA for examining the genotype of ILTVs<sup>[63]</sup>.

Oldoni *et al.*<sup>[51]</sup> (2007) investigated ILTV isolates from commercial poultry that were collected between 1988 and 2005 using multiple gene PCR-RFLP analysis (ORFB-TK, ICP4, UL47/gG, and gM/UL9). They were able to separate ILTVs into nine genetic groups. Group I and II comprised the USDA reference strain and TCO vaccine strains. Group IV isolates were identical to CEO vaccine strains, whereas group V isolates, which had one PCR-RFLP pattern different from the CEO vaccine strains were CEO-related isolates. Group VI, VII, VIII, and IX were field ILTV strains with genomic types different from CEO and TCO vaccines. These groups also showed different isolates based on pathogenicity. In that report, most of ILTV positive poultry isolates were related to vaccine strains<sup>[51]</sup>. Oldoni *et al.*<sup>[52]</sup> in 2008 investigated 46 ILTV field isolates collected in the US from 2006 to 2007. After multiple PCR-RFLP genotype analysis, many isolates were similar to vaccine strains<sup>[52]</sup>. According to these reports, most ILTV field isolates in the US might come from vaccine reversion. Differentiation of vaccine from field viruses is important since countries can initiate trade barrier for importation of chicken products from areas where virulent field viruses exist. Therefore, the more common mild ILT is commonly referred to a “vaccinal” ILT even though PCR-RFLP testing has not always been done.

In Europe, 104 field isolates were collected during 35 years from eight different countries. These virus isolates were analyzed with PCR-RFLP targeting the TK gene and it was shown that they separate into 3 genetic groups. It was also shown that 98 of these field isolates had the same RFLP patterns as vaccine strains<sup>[59]</sup>. In Australia, PCR-RFLP was used to analyze ILTV gG, TK, ICP4, ICP18.5 and ORFB-TK genes in 20 strains. These isolates could be discriminated into five genetic groups. Some isolates were closely related to vaccine strains<sup>[64]</sup>.

## IMMUNITY AND VACCINATION

ILT vaccine induces protection against challenge in 1 wk. Humoral immunity is not the major immune response against ILTV in chickens. Research verified the impor-

tance of cell-mediated immunity (CMI) in the resistance to ILTV. An experiment was designed in which chickens were bursectomized with cyclophosphamide and surgical methods to block the humoral immune responses. Vaccinated bursectomized chickens developed protective CMI responses against virulent ILTV challenges<sup>[16]</sup>. It confirmed that CMI was more important than humoral immunity. Furthermore, local CMI responses in the trachea produced protection from ILTV challenge in bursectomized chickens. Mucosal antibodies were not essential for resistance to challenge<sup>[65]</sup>.

Vaccination is effective in the prevent ILTV infection. However, ILT vaccine viruses can create latent infected carrier chickens. These latent carriers are a source for spread of virus to non-vaccinated flocks. Therefore, it is recommended that ILT vaccines be used only in endemic areas. The most currently used ILT vaccine strains are attenuated modified-live TCOs or CEOs viruses. Compared with protection afforded by TCO and CEO vaccines, there was no significant difference in the immunity of chickens at 10 wk post vaccination. However, when chickens over 20 wk of age were vaccinated, the CEO vaccines induced better protection than TCO vaccines<sup>[66]</sup>. Methods for live vaccine administration are eye drop, drinking water, and aerosol spray. The drinking water route poses some problems in that chickens might not receive enough viruses at the target organ (nasal epithelial cells) and drinking water quality varies between poultry houses. Thus, these birds may fail to develop protective immunity and may have rolling (continual) reactions<sup>[67]</sup>. On the other hand, with spray route, some chickens may develop severe reactions, because excess dosage of small droplets can penetrate deep into the respiratory tract<sup>[68]</sup>.

Reports have shown that modified-live vaccines increase their virulence by bird-to-bird passage. Serially passaged modified-live ILT vaccines *in vivo* for 35 generations. After the 6th passage, this vaccine strain produced severe clinical signs in challenged chickens. Furthermore, restriction endonuclease analysis of the viral genomes between original and final passage showed no differences between isolates. CEO vaccines have the tendency to increase in virulence more than TCO vaccines, when passed in chickens<sup>[69,70]</sup>. Investigations of ILTV isolates collected from around the world were analyzed by PCR-RFLP. They revealed that some current field virulent isolates were closely related to vaccine strains. This implies that field isolates originated from vaccine strains after back passage in chickens<sup>[43,52,57-60]</sup>.

Recently recombinant vaccines have been commercialized. Including partial ILTV genes were inserted into fowlpox and HVT modified genomes. A recombinant fowlpox vaccine, which contained ILTV glycoprotein B (gB) gene, was shown to induce protection against virulent strains<sup>[71]</sup>. Another recombinant fowlpox virus, which contains ILTV gB and UL 32 genes, showed some efficacy to provide protection against virulent strain challenge *via* wing web administration<sup>[72]</sup>. Two licensed commercial recombinant ILT vaccines are used in the US. One is pro-

duced by the CEVA (Biomune Company, Lenexa KS), which uses fowl poxvirus as a vector with an insertion of ILTV gB and UL 32 genes. The other is produced by Intervet (Intervet, Inc. Millsboro, DE), in which ILTV gI and gD genes are cloned into HVT. When these licensed commercial recombinant ILT vaccines were vaccinated by 18-d-old embryos *in ovo* injection, they reduced the clinical signs, but not virus replication after challenge<sup>[73]</sup>. These recombinant ILTV vaccines did not cause latent infections and virulent reversion. Although these recombinant vaccines are safer than previously developed live vaccines, their increased cost and the fact that they must be injected has limited their use.

Several studies tried to develop new ILT vaccine candidates by gene deletion. Some ILTVs, with deleted virulent viral genes, retained their ability to induce immune responses without producing clinical signs and latency. Recombinant virus with deleted gJ, TK, and, UL0 genes readily showed attenuation, and could be used for vaccine production<sup>[74-76]</sup>. The gG-deficient ILTV, administered by either eye drop or drinking water routes for 3-wk-old specific-pathogen-free birds, induce adequate immunity against challenge. Therefore, it may be able to a use for large-scale vaccination. However, further studies need to be done to determine the protection of this gG-deficient vaccine on commercial chicken farms<sup>[77]</sup>. There were also ILTV non-essential genes, which were deleted to test their ability as vaccines. The ILT mutants, which had five unique ORF A-E deleted, removed gN and gM, and the green fluorescent protein was inserted into the UL50 gene deleted region<sup>[78-80]</sup>. The gC deleted ILTV resulted in reduced virulence and it could be a marker vaccine<sup>[81]</sup>. These recombinant and gene-deleted ILTVs could be used as candidates to differentiate vaccinated from field-infected birds. Recombinant vaccines used ILTV as a viral vector to contain H5 or H7 genes of highly pathogenic AIVs. These recombinant ILTV may protect birds from ILT and pathogenic AIV<sup>[76,82]</sup>. Recently, a new ILTV vector had a HPAI H5 gene inserted into a deleted UL50 gene region. This recombinant virus protected birds from homologous and heterologous H5N1 and H5N2 viruses challenge<sup>[83,84]</sup>. An ILTV gB gene DNA vaccine was developed. The gB gene combined with chicken IL-18 as bicistronic vector induced better protection in chicken from ILTV challenge than the gB gene monocistronic vector alone<sup>[85]</sup>. Using ILTV gB gene plasmid DNA vaccine and chicken IL-18 plasmid DNA as an adjuvant induced T helper-1 immune response, which protected birds from virulent ILTV challenge<sup>[86]</sup>.

### Prevention and control of ILTV infection using chicken house management

It is important to avoid contact between vaccinated or recovered field virus infected birds with non-vaccinated chickens. It is also critical to remove contaminated fomites for prevention and control of ILTV infection. To control ILTV outbreaks, improved biosecurity and management practices are necessary. Biosecurity includes

protocols and procedures to prevent pathogens from infecting and transmitting disease by humans, insects, wild birds, or other animals<sup>[87]</sup>. A study found that heating litter at 38 °C for 24 h, using commercial litter treatments, and in-house composting for 5 d reduced this virus below detection levels<sup>[88,89]</sup>. Rapid diagnosis, a suitable vaccination procedure, and co-operation between government and industry are critical for ILT control. In 2005, California had a “vaccinal LT” outbreak in broiler farms. Although the companies improved the biosecurity and vaccination in these farms, they did not stop the chickens from being infected with ILT. Therefore, a strategy of depopulation, extended downtime, and strict biosecurity eliminated ILT in these farms was performed<sup>[90]</sup>. Recently, for controlling the outbreaks, geographic information systems were used to provide the information of the regions for biosecurity, quarantine, vaccination, and the route to processing plants. Government agents, industry companies, growers, and veterinarians need to work together and design a program for outbreak control<sup>[91]</sup>.

## CONCLUSION

ILT continues as an economical important poultry disease. House management and biosecurity measures should be performed for disease control. For eradication ILT, the modified-live vaccines need to be replaced by improved recombinant vaccines for the prevention of latent infection and virulent reversion.

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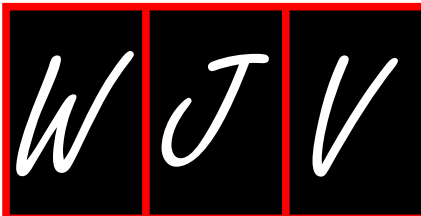
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We acknowledge our sincere thanks to our reviewers. Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of our World Series Journals. Both the editors of the journals and authors of the manuscripts submitted to the journals are grateful to the following reviewers for reviewing the articles (either published or rejected) over the past period of time.

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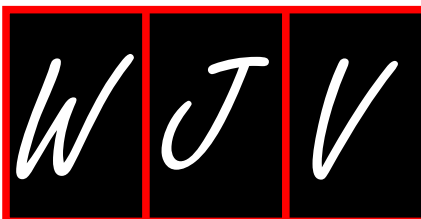
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## Events Calendar 2012

January 13-14, 2012

Innovation in Severe Acute Respiratory Infections  
Sitges, Spain

January 20, 2012

Exploiting Bacteriophages for Bioscience, Biotechnology and Medicine  
Welwyn Garden City,  
United Kingdom

January 20-22, 2012

International Science Symposium on HIV and Infectious Diseases  
Chennai, India

February 26-29, 2012

10th American Society for Microbiology Biodefense and Emerging Diseases Research Meeting  
Washington, DC, United States

March 11-15, 2012

8th International Symposium on Pneumococci and Pneumococcal Diseases  
Foz de Iguaçu City, Brazil

March 12-16, 2012

Vaccine World Summit India  
Hyderabad, India

March 14-17, 2012

22nd Annual Meeting of the German Society for Virology  
Essen, Germany

March 21-26, 2012

2012 HIV Vaccines  
Keystone, CO, United States

March 21-26, 2012

Viral Immunity and Host Gene

Influenza

Keystone, CO, United States

March 26-31, 2012

Cell Biology of Virus Entry, Replication and Pathogenesis  
Whistler, BC, Canada

March 26-31, 2012

Frontiers in HIV Pathogenesis, Therapy and Eradication  
Whistler, BC, Canada

April 20-21, 2012

2012 Molecular Virology Workshop  
Daytona Beach, FL, United States

April 22-25, 2012

2012 28th Clinical Virology Symposium  
Daytona Beach, FL, United States

April 24-27, 2012

European Molecular Biology Organization Workshop - Antigen presentation and processing  
Amsterdam, Netherlands

May 19-22, 2012

European Molecular Biology Organization and European Molecular Biology Laboratory Symposium - New perspectives on immunity to infection  
Heidelberg, Germany

June 11-16, 2012

Antiviral RNAi: From Molecular Biology Towards Applications  
Pultusk, Poland

June 28-29, 2012

7th International Workshop on Hepatitis C - Resistance and New

Compounds

Cambridge, MA, United States

July 16-20, 2012

European Molecular Biology Organization Conference Series - Viruses of microbes: From exploration to applications in the -omics era  
Brussels, Belgium

July 19-20, 2012

7th International Workshop on HIV Transmission - Principles of Intervention  
Washington, DC, United States

July 21-25, 2012

31st Annual Meeting of American Society for Virology  
Madison, WI, United States

July 30 - August 1, 2012

3rd Annual Symposia of Hepatitis Virus  
Guangzhou, China

August 20-22, 2012

2nd World Congress on Virology  
Las Vegas, NV, United States

September 7-9, 2012

Viral Hepatitis Congress 2012  
The Johann Wolfgang Goethe University, Frankfurt, Germany

October 18-20, 2012

2nd World Congress on Controversies in the Management of Viral Hepatitis (C-Hep)  
Berlin, Germany

November 28 -December 1, 2012

6th Asian Congress of Paediatric Infectious Diseases  
Colombo, Sri Lanka

## GENERAL INFORMATION

*World Journal of Virology* (*World J Virol*, *WJV*, online ISSN 2220-3249, DOI: 10.5501) is a bimonthly peer-reviewed, online, open-access (OA), journal supported by an editorial board consisting of 137 experts in virology from 41 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJV* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJV* is an OA journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJV* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an

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### Aims and scope

*WJV* aims to report rapidly new theories, methods and techniques for prevention, diagnosis, treatment, rehabilitation and nursing in the field of virology. *WJV* covers topics concerning arboviral infections, bronchiolitis, central nervous system viral diseases, DNA virus infections, encephalitis, eye infections, fatigue syndrome, hepatitis, meningitis, opportunistic infections, pneumonia, RNA virus infections, sexually transmitted diseases, skin diseases, slow virus diseases, tumor virus infections, viremia, zoonoses, and virology-related traditional medicine, and integrated Chinese and Western medicine. The journal also publishes original articles and reviews that report the results of virology-related applied and basic research in fields such as immunology, physiopathology, cell biology, pharmacology, medical genetics, and pharmacology of Chinese herbs.

### Columns

The columns in the issues of *WJV* will include: (1) Editorial: To introduce and comment on the substantial advance and its importance in the fast-developing areas; (2) Frontier: To review the most representative achievements and comment on the current research status in the important fields, and propose directions for the future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (6) Review: To systemically review the most representative progress and unsolved problems in the major scientific disciplines, comment on the current research status, and make suggestions on the future work; (7) Original Articles: To originally report the innovative and valuable findings in virology; (8) Brief Articles: To briefly report the novel and innovative findings in virology; (9) Case Report: To report a rare or typical case; (10) Letters to the Editor: To discuss and make reply to the contributions published in *WJV*, or to introduce and comment on a controversial issue of general interest; (11) Book Reviews: To introduce and comment on quality monographs of virology; and (12) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on the research in virology.

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All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

### Biostatistical editing

Statistical review is performed after peer review. We invite an expert in Biomedical Statistics to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Redit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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In the interests of transparency and to help reviewers assess any potential bias, *WJV* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: [http://www.icmje.org/ethical\\_4conflicts.html](http://www.icmje.org/ethical_4conflicts.html).

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### Statement of informed consent

Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors

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When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

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**Title:** Title should be less than 12 words.

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### Abstract

There are unstructured abstracts (no less than 256 words) and structured abstracts (no less than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no less than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g.  $6.92 \pm 3.86$  vs  $3.61 \pm 1.67$ ,  $P < 0.001$ ; CONCLUSION (no more than 26 words).

### Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

### Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: [http://www.wjgnet.com/2220-3249/g\\_info\\_20100725072755.htm](http://www.wjgnet.com/2220-3249/g_info_20100725072755.htm).

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

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Data that are not statistically significant should not be noted. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 are used. A third series of *P* values can be expressed as <sup>e</sup>*P* < 0.05 and <sup>f</sup>*P* < 0.01. Other notes in tables or under illustrations should be expressed as <sup>1</sup>F, <sup>2</sup>F, <sup>3</sup>F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

## Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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### Format

#### Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

### Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

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Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

### Statistical data

Write as mean  $\pm$  SD or mean  $\pm$  SE.

### Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

### Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pres-

sure,  $p$  (B) = 16.2/12.3 kPa; incubation time,  $t$  (incubation) = 96 h, blood glucose concentration,  $c$  (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration,  $p$  (CEA) = 8.6 24.5  $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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### Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

### Italics

Quantities:  $t$  time or temperature,  $c$  concentration,  $A$  area,  $l$  length,  $m$  mass,  $V$  volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, *etc.*

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, *etc.*

Biology: *H. pylori*, *E. coli*, *etc.*

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