The Journal of Basic & Applied Zoology (2014) 67, 119–126

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The Journal of Basic & Applied Zoology

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Serological, molecular characterization and epidemiological situation of equine influenza in the Arabic Maghreb countries between 1972 to 2010

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Received 10 August 2013; accepted 18 December 2013
Available online 30 January 2014

KEYWORDS

Equine influenza;
Influenza virus;
Predivergence phase;
Eurasian lineage

Abstract Equine influenza is an infectious and contagious disease of horses. Studies on this topic are rare in the Maghreb countries. Therefore, the aim of this work is to present the various studies conducted on serological and molecular equine influenza virus since 1972 in the Maghreb region in particular in Morocco, Algeria and Tunisia.

A total of four equine influenza strains were isolated in the Maghreb Arab region. A/equine/Nador/1/1997(H3N8), A/equine/Essaouira/2/2004(H3N8), A/equine/Essaouira/3/2004(H3N8) and A/equine/Algiers/1/1972(H3N8).

The highest homology of HA nucleotide sequences of A/equine/Nador/1/1997(H3N8) with European strains: A/equine/Italy/1199/1992(H3N8) and A/equine/Brescia/1999(H3N8) clearly clustered A/equine/Nador/1/1997(H3N8) with the strains belonging to the European lineage. However, A/equine/Algiers/1/1972(H3N8), A/equine/Essaouira/2/2004(H3N8) and A/equine/Essaouira/3/

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Peer review under responsibility of The Egyptian German Society for Zoology.

http://dx.doi.org/10.1016/j.jobaz.2013.12.001

2004(H3N8) were placed in the predivergent lineage indicating that like-Miami/63 strains infected equids in Morocco in 2004.

This finding does not corroborate the recent studies of the H3N8 subtype of equine influenza viruses which have demonstrated that the oldest equine H3N8 strains, circulating before 1990 apparently went extinct.

Contents

Background

Equine influenza (EI) is a highly contagious respiratory viral disease of horses, donkeys and their cross products caused by RNA virus (Gurkirpal, 1997; Barquero et al., 2007; Virmani et al., 2010). It generates significant economic losses, often due to the ineffectiveness of vaccines and lower sportive performance of horse (Lai et al., 2004; Lewis et al., 2011). Two subtypes of equine influenza virus H7N7 and H3N8, were identified: (prototypes A/equine/Prague/1/56(H7N7) and A/equine/Miami/1/63(H3N8) (Sovinova et al., 1958; Waddell et al., 1963).

The first subtype (H7N7) is antigenically stable. Since 1979, no outbreaks have been reported and only some serological traces in unvaccinated horses indicate that this virus still circulates as subclinical form (Zientara, 2001; Daly et al., 2004).

While, the second subtype (H3N8) continues to circulate worldwide, and was responsible of all recent reported outbreaks. Phylogenetic analysis of different strains isolated since 1989 indicates that the virus had evolved into two antigenic distinct lineages: Eurasian and American lineages (Kawaoka et al., 1998).

The last lineage subsequently diverged into three sublineages: South America, Florida and Kentucky (Lai et al., 2004). Currently, the sublineage Florida had evolved into two clades 1 and 2.

The viral particles are highly pleomorphic, spherical shape (with a diameter of 80–120 nm), ovoid or filamentous (Nayak and Baluda, 1967; Burnouf et al., 2004). Two major glycoproteins are present on the surface of the virus: hemagglutinin (HA) and neuraminidase (NA). HA plays a major role in the determination of the host specificity and consists of two polypeptide subunits; HA1 of 329 amino acids (50 kDa) and HA2 of 221 amino acids (25KDa), which are linked by a single disulfide bond between HA1 14 and HA2 137 (Bullough et al., 1994). HA1 subunit is the center of minor mutations "drift", particularly in the five antigenic sites (A, B, C, D and E), while the HA2 generally shows a high degree of conservation and plays a critical role in the delivery of the viral nucleocapsid into the host cell (Varea kova et al., 2008).

Severe epizootics with high morbidity occurred mainly in Europe and America due to minor mutations "drift" in the antigenic sites of the subunit (HA1). Disturbingly, the new strains were partially recognized by preexisting antibodies and vaccines became ineffective (Barbic et al., 2009).

According to the meeting (February 27, 2012) of the expert group of the World Organization for Animal Health (OIE), who are in charge for monitoring the composition of vaccines against EI: several outbreaks have been reported in Europe, America and Asia during the year 2011. The sequencing of HA1 genes of isolated viruses showed their relationship with the American lineage (Florida sublineage clades 1 and 2) (http://www.oie.int/fr/notre-expertise-scientifique/informations-specifiques-et-recommandations/grippe-equine/).

Despite its impact on the equine industry worldwide, the studies on EI are rare in the Arabic Maghreb. This article will briefly present the results of different serological and molecular studies carried out on equine influenza in Morocco, Tunisia and Algeria. Studies on this subject are unavailable in Mauritania and Libya.

Epidemiological situation in Arabic Maghreb

Morocco

Like other Maghreb countries, the studies on EI are rare in Morocco. However, serological studies conducted since 1978 revealed the existence of the co-circulation of two subtypes H7N7 and H3N8 with the presence of mixed infection (Fontaine and Moraillon, 1980).

From 1990 to 1994, a seroepidemiological survey was carried out and the results showed a predominance of subtype

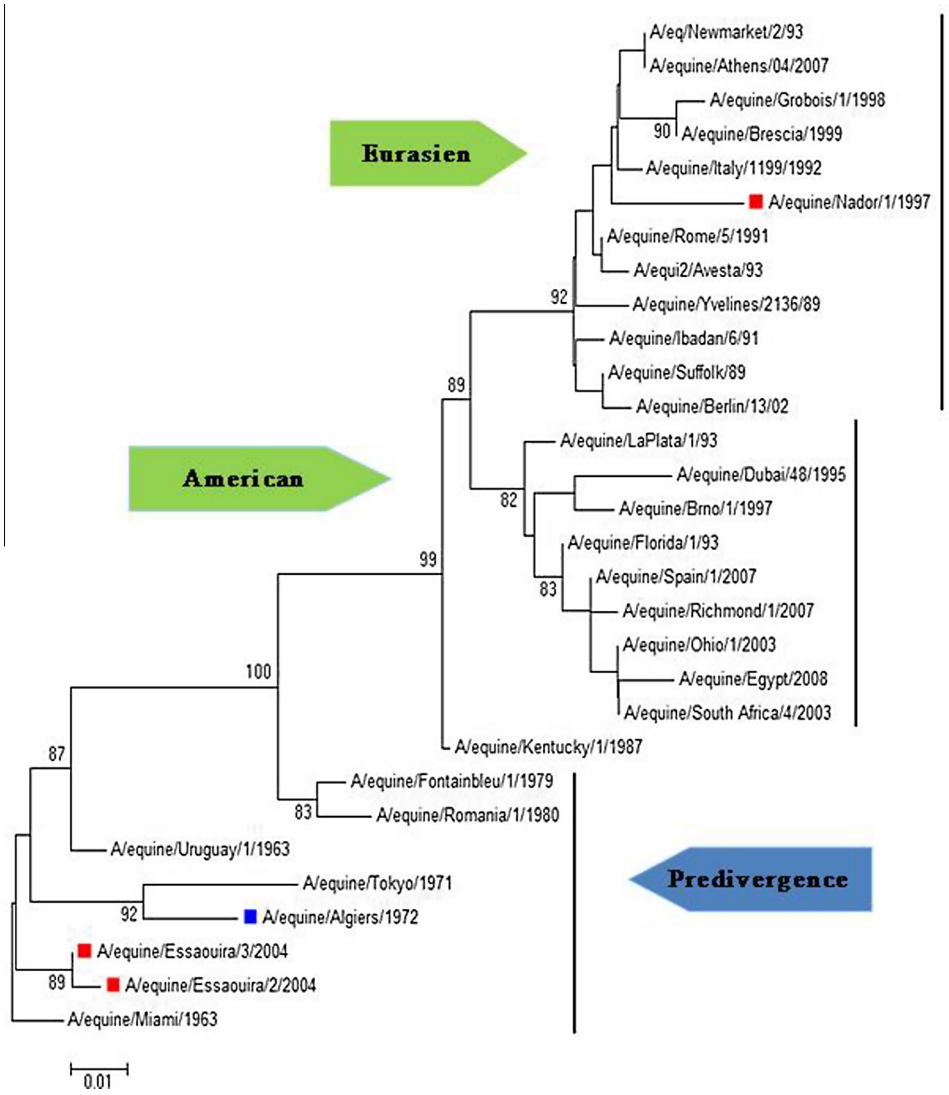

Figure 1 Phylogenetic tree obtained by comparing amino acid sequences for 30 strains derived from the hemagglutinin gene HA1 (1–329 aa) using the Neighbor Joining method (Saitou and Nei, 1987) in which the A/equine/Miami/63 HA sequence as the root. The tree was visualized using the MEGA5.1 software (Tamura et al., 2007). AAA43164; A/equine/Uruguay/1/1963, AAA43114; A/equine/Tokyo/1971, AAA43111; A/equine/Algiers/1972, ACF22126; A/equine/Fontainbleu/1/1979, ACD85396; A/equine/Romania/1/1980, ACD85374; A/equine/Kentucky/1/1987, ACA24568; A/equine/Suffolk/89, CAA48482; A/equine/Yvelines/2136/89,BAA33940; A/equine/Ibadan/6/91, CAA64893; A/equine/Rome/5/1991, ACD85341; A/equine/Italy/1199/1992, ACD85308; A/equine/Florida/1/93, AAB36978; A/equine/LaPlata/1/93,BAA33947; A/equi 2/Avesta/93, CAA74385; A/eq/Newmarket/2/93, CAA59416; A/equine/Dubai/48/1995, AEI26218; A/equine/Nador/1/1997, AFJ69903; A/equine/Grobois/1/1998, ACH95594; A/equine/Ohio/1/2003, ABA39846; A/equine/Essaouira/3/2004, AFJ69909; A/equine/Spain/1/2007, ADO78886; A/equine/Egypt/6066NAMRU3-VSVRI/2008 (A/equine/Egypt/2008), ACH95682; A/equine/Essaouira/2/2004, AFJ69905; A/equine/Athens/04/2007, ADF55752; A/equine/Berlin/13/02, ABP35588; equine/Brescia/1999, ABU46321; A/equine/Brno/1/1997, AEI26221; A/equine/South Africa/4/2003, ADB45165; A/equine/Richmond/1/2007, ACH95569.

H7N7 with seroprevalence rates of approximately 16.9% against only 8.6% for the subtype H3N8 (Elharrak et al., 1998).

In December 1997, the first virus isolation was performed in a mule in the north of Morocco with acute respiratory signs, this strain is baptized A/Equi2/Maroc/97 (Actually called, A/equine/Nador/1/1997) (Kissi et al., 1998).

In 2004, several outbreaks of EI were reported in several parts of the country (Essaouira, Khouribga and Larache). Three equine species had been affected with 70 cases of morbidity and mortality cases (one death case of a donkey). In contrast of the epizootic of 1997, the 2004 epizootic was notified to the OIE (World Organization for Animal Health, 2004). In June 2004, strains A/equine/Essaouira/2/2004 and A/equine/Essaouira/3/2004 were isolated from a horse and a donkey, respectively.

HA nucleotide sequences of the Moroccan equine influenza isolates were delineate and submitted to the GenBank by Boukharta et al., 2012 (http://www.ncbi.nlm.nih.gov/). The accession numbers of the A/equine/Nador/1/1997, A/equine/Essaouira/2/2004 and A/equine/Essaouira/3/2004 partial HA genes are numbered JQ955607, JQ955609 and JQ955612, respectively.

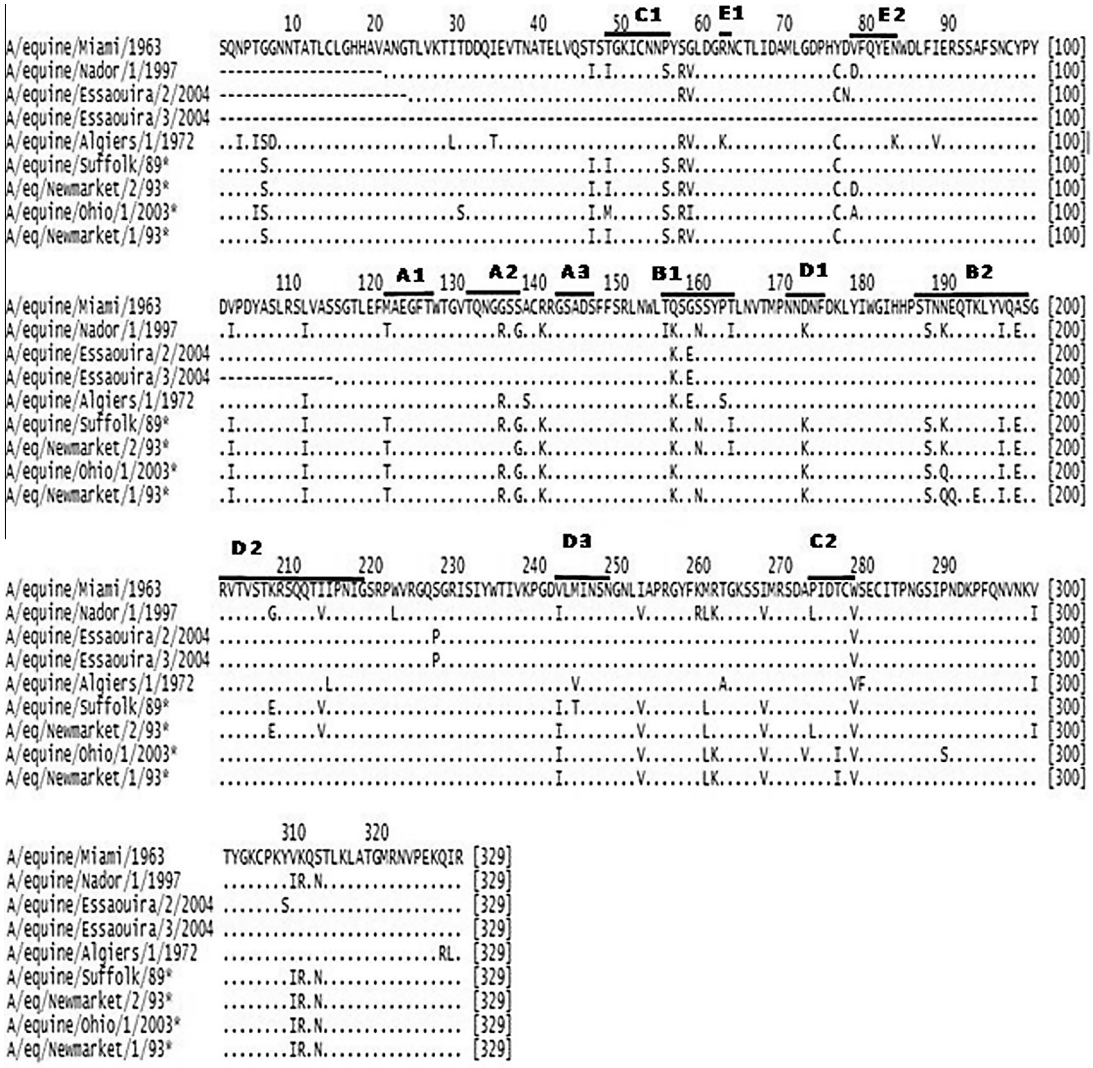

Figure 2 Amino acid alignment of predicted HA1 sequences compared to A/equine/Miami/1963: **Accession numbers**: A/equine/Miami/1963, AAA43164; A/equine/Nador/1/1997, AFJ69903; A/equine/Essaouira/2/2004,AFJ69905; A/equine/Essaouira/3/2004, AFJ69909; A/equine/Algiers/1/1972, ACF22126; **Vaccine strains**: (A/equine/Suffolk/89, CAA48482; A/eq/Newmarket/2/93, CAA59416) (Eurasien Lignage); (A/equine/Ohio/1/2003, ABA39846; A/eq/Newmarket/1/93, Q82846) (Americain Lignage) (*commercialized vaccine strains in Morocco). (Positions antigenic sites: C1 (48, 55), C2 (273,278), E1 (62, 63), E2 (78, 83), A1 (121,126), A2 (131, 137), A3 (142, 146), B1 (155, 163), D1 (170, 174), B2 (186, 199), D2 (201, 218), D3 (242, 248)) (Both et al., 1983; Martella et al., 2007; Nakajima et al., 2004; Underwood, 1982; Virmani et al., 2010) (*commercialized vaccine strains in Morocco). Alignment result was visualized using the MEGA5.1 software (Tamura et al., 2007)).

Phylogenetic and antigenic analyses of the three strains reported in Morocco between 1997 and 2004, are elucidated. Both strains of A/equine/Essaouira/2/2004 and A/equine/Essaouira/3/2004 belong to an evolutionary phase of predivergence, while the strain A/ equine/Nador/1/1997 belong to the Eurasian lineage (Fig. 1). This finding does not corroborate the recent studies of the H3N8 subtype of equine influenza viruses which demonstrated that the oldest equine influenza H3N8 strains (like-Miami), circulating before 1990 apparently went extinct (Martella et al., 2007).

In 2010, Boukharta et al. (2012) conducted a serological study of 726 horses, the results showed an overall seroprevalence of approximately 52% and co-circulation of two viral subtypes H7N7 and H3N8. In Africa, traces of antibodies against subtype H7N7 have also been reported in Mali (Sidibé et al., 2002). The presence of serological traces against the H7N7 subtype in unvaccinated equids can be attributed to the serological techniques used or the persistence of antibodies; further investigations are needed to confirm its circulation in the region.

Analysis of the amino acid sequences of the HA1 subunit of three Moroccan strains revealed that the strain A/equine/Nador/1/1997 shows six mutations comparing with the A/eq/Newmarket/2/93 (Gly/135/Arg, Thr/155/Ile, Glu/207/Gly, Trp/222/Leu, Lys/259/Arg, Arg/261/Lys). Whereas both strains: A/equine/Essaouira/2/2004 and A/equine/Essaouira/3/2004 have three mutations at antigenic sites of HA1 (Gln/156/Lys, Gly/158/Glu, Trp/278/Val) when compared to the prototype equine H3N8 influenza virus (A/equine/Miami/63) (Fig. 2, Table 1).

Algeria

The first declared outbreak in Algeria was in 1971–1972 after the onset of acute signs of respiratory infection in equids in Algiers, the causative virus was isolated and found to belong to subtype (H3N8) (Benmansour et al., 1977) and successively the sequencing of A/equine/Algiers/1/1972 (H3N8) strain was carried. The HA nucleotide sequence is available in the GenBank under the accession number CY032945.

This strain belongs to the predivergence evolutionary phase (Fig. 1). While the analysis of amino acid sequences of the HA1 subunit of strain A/equine/Algiers/1/1972 shows four mutations at antigenic sites of HA1 compared to A/equine/Miami/63 (Gly/135/Arg, Gln/156/Lys, Gly/158/Glu, Trp/278/Val) (Table 1).

Since the first isolation of the Algerian strain, two seroepidemiological surveys were conducted by Bererhi et al. from January to December 2006 and Laabassi et al. between May 2009 January 2010, respectively (Bererhi et al., 2009; Laabassi et al., 2012). The first study concerned an effective of 132 unvaccinated equidae (60 donkeys and 72 horses) in Khenchela region (North-East), the results show an overall prevalence rate of 24.24% (32/132) and co-circulation of the two subtypes (H7N7) and (H3N8) with the presence of mixed infection. Depending on the species, donkeys were more affected (31.66%) and only (18.05%) in horses (Bererhi et al., 2009).

The second survey was carried out on 297 horses stationed in the region of western Algeria (Tiaret) and in eastern Algeria (regions Barika and El Eulma). The use of the hemagglutination inhibition (HI) method allowed the detection of 164 (55.2%) positive samples with H3N8/Russie/1983, 86 (29.0%) with Miami/1963 (H3N8) strain and 5 (1.7%) with Prague/1956 (H7N7) (Laabassi et al., 2012).

At the end of the second study, two important points for epidemiological genetic evolution of EI viruses in Algeria and neighboring countries were raised:

- Firstly, there is a difference of seropositivity between the strains: H3N8/Russie/1983 and H3N8/Miami/1963 with a better affinity for H3N8/Russie/1983 strain (strain apparently antigenically far from the reference strain (A/equine/Miami/63)).
- Secondly, the seroprevalence of EI evaluated by HI via Russie/1983 (H3N8) strain was significantly more frequent in the West (Tiaret region) than eastern Algeria (El-Eulma (wilaya of Setif) and especially Barika (Batna wilaya). According to the author, this disparity is likely due to the circulation of strains in border neighboring countries, particularly in Tunisia in the East and Morocco in the West of Maghreb (Laabassi et al., 2012).

Tunisia

The first outbreak was reported in 1978–1979. A few months later, a seroepidemiological survey was carried out by Ellouze (1980) and showed that the responsible virus seropositivity was the subtype H7N7 with an overall rate of (65%). In 1994, Bousseta et al. (1994) conducted a survey on 313 unvaccinated horses and 41 donkeys, concluding that the two subtypes (H7N7) and (H3N8) were circulated with a positivity rate of

Table 1 Comparison of mutations in five antigenic sites of HA1 subunit.

Antigenic sites	Site C		Site E	Site A		Site B										Site D						Site C			
	C1	C1	E2	A1	A2	A2	B1	B1	B1	B1	B1	B2	B2	B2	B2	B2	B2	D1	D2	D2	D3	C2	C2	C2
	T	P	V	M	G	S	T	Q	G	S	T	N	E	K	V	A	K	D	K	V	I	L	T	w
	48	55	78	121	135	137	155	156	158	159	163	187	189	190	193	196	198	172	207	213	242	273	276	278
A/equine/Miami/1963																								
A/equine/Nador/1/1997	I	S	D	T	R	G	I	K	.	N	I	S	K	.	.	I	E	K	G	V	I	L		V
A/equine/Essaouira/2/2004	.	.	.	.	.	.	.	K	E	.	.	.	.	.	.	.	.	.	.	.	.	.		V
A/equine/Essaouira/3/2004	–	–	–	.	.	.	.	K	E	.	.	.	.	.	.	.	.	.	.	.	.	.		V
A/equine/Algiers/1/1972	.	.	.	.	R	.	.	K	E	.	.	.	.	.	.	.	.	.	.	.	.	.		V
A/equine/Suffolk/89*	I	S	.	T	R	G	.	K	.	N	I	S	K	.	.	I	E	K	E	V	I	.		V
A/eq/Newmarket/2/93*	I	S	D	T	.	G	.	K	.	N	I	S	K	.	.	I	E	K	E	V	I	L		V
A/equine/Ohio/1/2003*	M	S	A	T	R	G	.	K	.	.	.	S	Q	.	.	I	E	K	.	.	I	.	I	V
A/eq/Newmarket/1/93*	I	S	.	T	R	G	.	K	.	N	.	S	Q	Q	E	I	E	K	.	.	I	.	I	V

* Commercialized vaccine strains in Morocco.

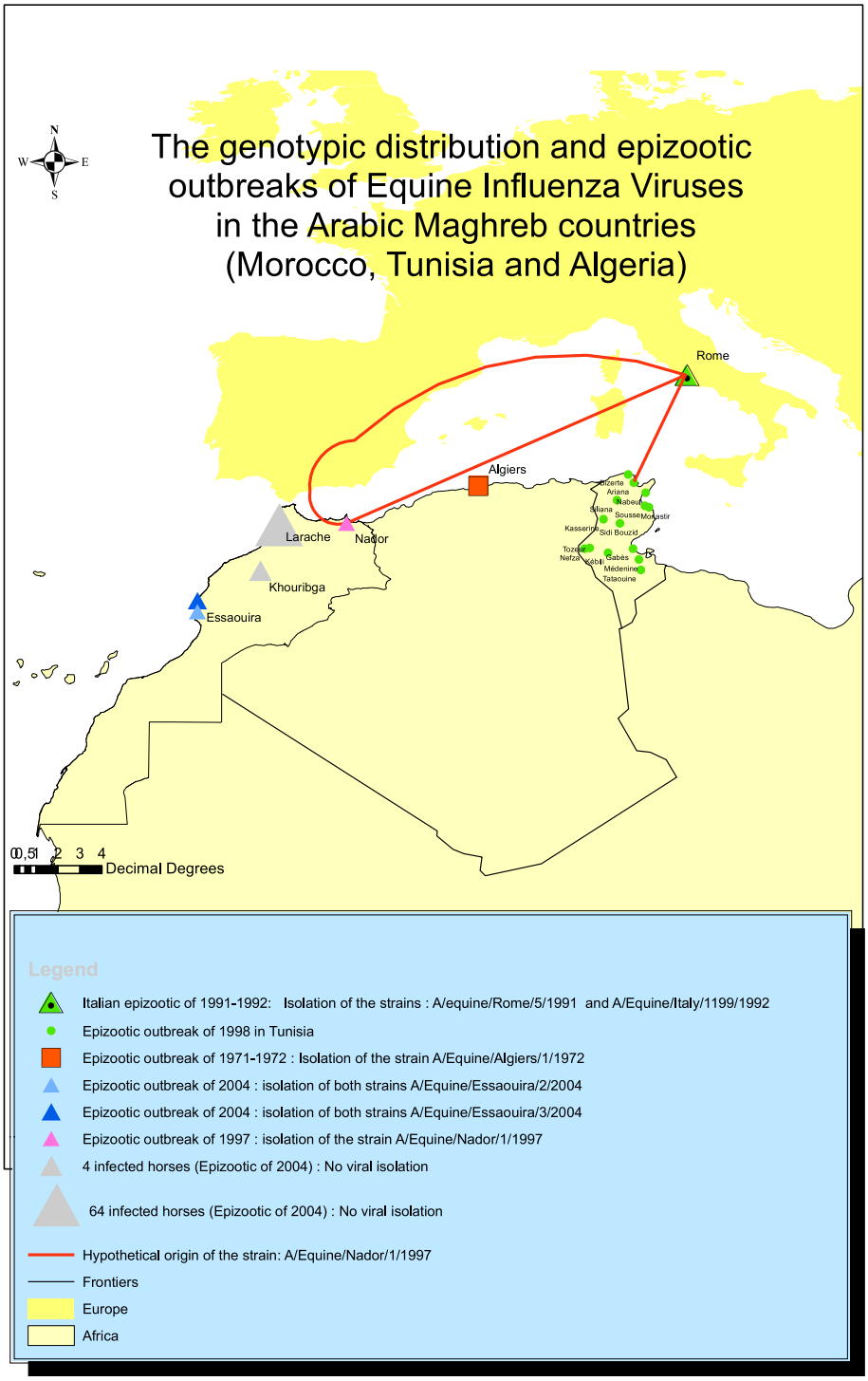

Figure 3 The genotypic distribution and epizootic outbreaks of equine influenza viruses in the Arabic Maghreb countries (Morocco, Tunisia and Algeria).

4% and 2.8%, respectively (HI titer of 40). Subsequently, different results were reported by Baazaoui in 1996, with a positivity rate of 10% for subtype H7N7 and 0.9% for subtype H3N8 (Baazaoui, 1996).

In January 1998, an epizootic of EI occurred in Tozeur and Nafta in southern Tunisia among line equids (horses 70%, 12% and 18% of donkeys and mules) showed signs of EI disease. Until July by the same year, more than 94 outbreaks have been identified and more than 1542 horses have been declared infected in 13 governorates (Ariana, Bizerte, Gabes, Kasserine, Kebili Medenine, Nabeul, Sidi Bouzid, Siliana, Sousse, Monastir, Tataouine Nefza) (Chabchoub et al., 2001).

In November 1998, after the first outbreak, a new outbreak was reported in the governorate of Ariana (northeast) among 109 race horses, of which 90 were previously vaccinated during the month of June–July 1998. It is noteworthy to mention that

among 11 sera from unvaccinated horses tested against A/equine/Nador/1/1997 strain (previously isolated in 31 December 1997 in Morocco), nine (9) were positive and showed very high responses (HI titer of 80–1280) (Chabchoub et al., 2001).

Due to their geographical location, which is close to the coastal city of Bizerte (54 km), representing the main commercial port of maritime traffic, especially with Europe and taking into account the results of the phylogenetic analysis carried out by Moroccan strains, it seems that the first outbreak of the disease was in the region of Ariana that has infected all of Tunisia in 1998 and originally the EI virus strains were circulated in Italy in the 1990s (i.e.: A/equine/Rome/5/1991 and A/equine/Italy/1199/1992) (Fig. 3).

Concluding remarks

In terms of conclusion and the outcome of various studies conducted on serological and virological investigations in the Arabic Maghreb, especially Morocco, Algeria and Tunis, we have noted the following five points:

- The hemagglutination inhibition (HI) test is used by many authors in Arabic Maghreb countries for detection of antibodies to influenza viruses. This test HI is rapid with good sensitivity, and also has a reproducibility of 84% to 96% for myxoviruses (Boliar et al., 2006). For many of serological studies, the technique used to eliminate nonspecific inhibitors from horse serum is heat treatment. However, this treatment may give false positive results.
- Several authors have reported the co-circulation of two subtypes (H7N7) and (H3N8) with the proportions of variable seropositivity. According to the different serological results, it seems that two evolutionary phases can be distinguished: the first phase was between 1978 and 1997 and there was a predominance of subtype (H7N7) and the second phase was from 1998 to 2010, in which subtype (H7N7) was supplanted by the subtype (H3N8).
- The affinity of the sera antibodies obtained from unvaccinated equidae decreases against the reference strain A/equine/Miami/1/1963; this clearly shows that new strains are in circulation in the Arab Maghreb region. These circulating strains are obtained either due to minor mutations or indigenous strains imported from Europe (closest region), like the case for the strain A/equine/Nador/1/1997.
- The mules and donkeys species seem to play an important role in the epidemiology of EI. Consequently, serological traces of two subtypes (H7N7) and (H3N8) have been reported in those species in different studies, especially, strain A/equine/Nador/1/1997 that was isolated from a mule.
- Understanding the mechanism of EI evolution in the Arabic Maghreb region must go through a comprehensive approach that includes the five Maghreb countries (determining places of high contamination, potential relationship between the virus and the environment, etc..) and the great risk posed by the European and American strains that are antigenically more advanced than local strains.

Indeed, the installation of a functional monitoring network could probably allow a genetic characterization in real time of circulated equine influenza virus strains in the region and provide adequate vaccine composition.

Acknowledgments

This study was supported by the University Hassan II. Mohammedia-Casablanca, Faculty of Sciences and Techniques, Mohammedia, Morocco. The authors would like to thank the Inspection de Service de Santé Militaire/Division Vétérinaire (EMG/ISS/DV) for their support.

Authors' contributions

The work presented here was carried out in collaboration between all authors. BM performed and wrote the first draft of the manuscript. FZ helped in redrafting the manuscript. AA designed the genotypic distribution map. MME, EHM, and TN conceived of the study and helped revising the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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