UNIVERSITÉ DE LIÈGE

FACULTÉ DE MÉDECINE VÉTÉRINAIRE

DÉPARTEMENT DE MORPHOLOGIE ET DE PATHOLOGIE

SERVICE DE PATHOLOGIE ET D'AUTOPSIE

La Mx1 bubaline *(Bubalus bubalis):* première contribution à sa détection et à l'examen de sa fonction antivirale

First contribution to water buffalo *(Bubalus bubalis)* type-I/III interferons - induced Mx1 detection and anti-viral activity

Đóng góp bước đầu trong phát hiện và xác định tính kháng virut của protein Mx1 ở trâu *(Bubalus bubalis)*

Phai DAM VAN

THESE PRESENTEE EN VUE DE L'OBTENTION DU GRADE DE

Docteur en Sciences Vétérinaires

ANNEE ACADEMIQUE 2019-2020

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DAM VAN Phai

ABBREVIATIONS

AA	Alopecia Areata
ARDs	Ankyrin-like Repeat Domains
ASF	American Swine Fever
bb	Bubalus bubalis
bbMx1	*Bubalus bubalis* Myxovirus Resistance Protein 1
BHV	Bovine Herpesvirus
BSA	Bovine Serum Albumin
bt	Bos taurus
BVD	Bovine Viral Diarrhoea
cDNA	complementary DNA
cf	Canis familiaris
DAPI	4′,6-diamidino-2-phenylindole
DNA	DeoxyriboNucleic Acid
DS	Down Syndrome
ec	Equus caballus
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FMD	Foot and mouth disease
HAT	Hypoxanthine-Aminopterin-Thymidine
HPAI	Highly Pathogenic Avian Influenza
HRP	Horseradish peroxidase conjugated
IBR	Infectious Bovine Rhinotracheitis
IFNs	Interferons
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISRE	Interferon-Stimulated Response Element
LACV	Lacrosse Virus
MDBK	Madin-Darby Bovine Kidney
ND	Newcastle disease
NP	Nucleoprotein
OD	Optical Density
OIE	World Organization for Animal Health
ORF	Open Reading Frame

PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline -Tween 20
PLM-NB	Promyelocytic Leukemia Protein-Nuclear Bodies
PRRS	Porcine reproductive & respiratory syndrome
RFLPs	Restriction Fragment Length Polymorphisms
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SFV	Semliki Forest Virus
SNPs	Single Nucleotide Polymorphism
ss	Sus scrofa
SSPE	Subacute Sclerosing Panencephalitis
TRPC	Transient Receptor Potential Canonical
VSV	Vesicular Stomatitis Virus

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Résumé

La protéine de résistance aux myxovirus 1 (Mx1) est une molécule intracellulaire induite par les interférons de type I/III conférant une propriété antivirale à l'hôte vis-à-vis de plusieurs virus. La protéine s'avère être un biomarqueur potentiel pour la détection d'infections virales précoces chez de nombreux vertébrés. Chez l'homme, les protéines MxA et MxB ont fait l'objet d'études approfondies pendant des décennies et il a été constaté que la concentration normale de la protéine MxA dans le sang périphérique de personnes en bonne santé était inférieure à 50 ng/ml, qu'elle était induite cliniquement peu de temps (1-2 heures) après l'exposition à une infection virale et qu'elle avait une demi-vie de 2-3 jours. La protéine MxA humaine présente dans le sang périphérique de patients s'est révélée être un biomarqueur sensible et spécifique des infections virales. La concentration sanguine en MxA est augmentée spécifiquement par l'infection par le rhinovirus, le virus respiratoire syncytial, le virus para-influenza, le virus de la grippe, le coronavirus et le métapneumovirus humain. En médecine humaine, la protéine MxA a été utilisée avec succès en tant que biomarqueur spécifique permettant de distinguer les infections virales des infections bactériennes, offrant ainsi des indications utiles pour le diagnostic et la prescription d'antibiotiques. Plusieurs études ont été menées sur la protéine Mx1 de nombreux animaux d'élevage, y compris les porcs, les chiens, les chevaux et les vaches.

Dans le but de mettre au point un nouvel outil de diagnostic efficace, peu coûteux et rapide, qui puisse être utilisé efficacement pour lutter contre les maladies virales en procédant au criblage en vue de la détection des buffles infectés par des virus, y compris des individus asymptomatiques, nous avons tout d'abord réussi à produire et à caractériser la protéine Mx1 (bbMx1) recombinante de buffle (*Bubalus bubalis*) et une série d'anticorps monoclonaux spécifiques de cette protéine.

Afin de produire la protéine recombinante bbMx1, deux vecteurs ont été synthétisés pour exprimer une Mx1 bubaline de référence (NP_001277782, SM1) dans *Escherichia coli* (nommé pET-28b [+] / His6x-bbMx1) ou dans des cellules de mammifère (pD657RA / V5-bbMx1). Dans les deux cas, la Mx1 recombinante bubaline a été intentionnellement fusionnée, en position N-terminale, soit à un tractus polyhistidine (pET-28b [+] - His6-bbMx1) soit à un épitope V5 (pDA657RA / V5-bbMx1). Dans ce dernier cas, la protéine V5-bbMx1 a été encore fusionnée à la protéine marqueur fluorescente RFP suivie du peptide 2A pouvant être clivé spontanément. La bbMx1recombinante a été exprimée avec succès dans des cellules d'*E. coli* et de mammifère par transfection transitoire à l'aide du réactif Lipofectamin 3000. Une analyse par SDS-PAGE du lysat cellulaire des populations cellulaires transfectées ou exposées au poly-i-c-révèle systématiquement une bande dont le poids moléculaire estimé vaut environ 75 kDa, soit le poids attendu de la Mx1 bubaline.

Les interférons de type I/III fournissent des mécanismes de défense intracellulaires innés puissants et universels contre les virus. Parmi les effecteurs antiviraux induits, les protéines Mx de certaines espèces apparaissent comme des composants clés de la défense contre les virus de la grippe

A. On s'attend à ce qu'une telle protéine antivirale possède une plate-forme dédiée à la reconnaissance des-dits virus. Ici, l'activité anti-influenza de bbMx1 et de quatre autres protéines Mx1 de mammifère distinctes a été mesurée en comparant le nombre de cellules positives à la nucléoprotéine virale 7 heures après l'infection dans un échantillon de 100 000 cellules susceptibles de contenir à la fois des cellules Mx-positives et Mx-négatives. La déplétion systématique ($p < 0,001$) des cellules virales positives à la nucléoprotéine parmi les populations de cellules exprimant Mx1 équine, bubaline, porcine et bovine par rapport aux cellules Mx-négatives suggère une forte activité anti-influenza A. En recherchant des éléments communs de signature anti-influenza dans la séquence de ces protéines Mx, nous avons constaté qu'un résidu aromatique aux positions 561 ou 562 de la boucle L4 semblait essentiel pour la fonction anti-influenza et/ou la spécificité de Mx1 chez les mammifères.

Summary

Myxovirus resistance protein 1 (Mx1) is a type I/III interferons-induced protein conferring the hosts an antiviral property against several viruses. The protein is also found to be a potential biomarker for early detection of viral infections in many vertebrates. In humans, MxA and MxB proteins have been studied intensively for decades and it has been found that the normal concentration of MxA protein in peripheral blood of healthy people is less than 50 ng/mL, that it is induced very shortly (1-2 hours) after exposure to viral infections and that it displays a half-life of 2-3 days. Human MxA protein in peripheral blood of patients has been shown to be clinically sensitive and specific biomarker for viral infections. The blood MxA concentration is increased specifically by infections by Rhinoviruses, respiratory syncytial viruses, parainfluenza viruses, influenza viruses, coronaviruses, and human metapneumoviruses. In human medicine, MxA protein has been used successfully as a specific biomarker for distinguishing viral to bacterial infections, providing good guidance for diagnostic and prescription of antibiotics. Several studies have been conducted on Mx1 protein of many livestock including pig, dog, horse and cow. However, there is no research conducted on the proteins Mx of water buffalo, even if this valuable ruminant, which is vulnerable to several dangerous viral diseases, has been increasingly economically important in Asia.

In order to produce recombinant bbMx1 protein, two vectors were synthesized for expressing a reference buffalo Mx1 either in Escherichia coli or in mammalian cells. In both cases, the recombinant water buffalo Mx1 was intendedly fused to a N-term functional group with either a poly-histidine tract or a V5 epitope. In the latter, the V5-bbMx1 protein was further fused to a N-term bipartite group consisting of the fluorescent marker protein RFP followed by the spontaneously cleavable peptide 2A. The recombinant bbMx1 was expressed successfully in both E. coli and mammalian cells by transient transfection using Lipofectamin 3000 reagent. SDS-PAGE analysis of cells lysate from transfected cell population and poly i:c -incubated buffalo cells illustrated of bbMx1 protein with molecular weight of almost 75kDa.

Type I/III interferons provide powerful and universal innate intracellular defense mechanisms against viruses. Among the antiviral effectors induced, Mx proteins of some species appear as key components of defense against influenza A viruses. It is expected that such an antiviral protein must display a platform dedicated to the recognition of said viruses. In my thesis, the anti-influenza activity of bbMx1 and other four distinct mammalian Mx1 proteins was measured by comparing the number of viral nucleoprotein-positive cells 7 hours after infection in a sample of 100,000 cells expected to contain both Mx1-positive and Mx1-negative cell subpopulations. The systematic depletion ($p < 0,001$) of virus nucleoprotein-positive cells among equine, bubaline, porcine and bovine Mx1-expressing cell populations compared to Mx-negative cells suggests a strong anti-influenza A activity. Looking for common anti-influenza signature elements in the sequence of these Mx proteins, we

found that an aromatic residue at positions 561 or 562 in the L4 loop seems critical for the anti-influenza function and/or specificity of mammalian Mx1.

GENERAL PREAMBLE

Domestic water buffalo is a valuable ruminant livestock playing crucial socio-economic and cultural roles in rural areas of Asian countries. However, they are not only the natural host of many viruses but also the subject of spreading several diseases to other livestock. In the recent decades, movements of the livestock species among farms, regions and countries has become increasingly vibrant over. Annually, millions of domestic water buffaloes have been commercially transported, contributing an important share to economic development of the corresponding areas. However, due to the limitations of the national and regional infectious disease control systems, especially the lack of effective, cheap and rapid tests for detection of early viral infections in the livestock prior to departure, the mis-transportation of buffaloes including asymptomatic individuals that carry pathogenic viruses are inevitable events, threatening the biosafety of the whole region.

Foot-and-mouth disease, for instance, has been circulating complicatedly throughout many Asian countries for several years, causing serious economic losses, even if it has been received overwhelming attention through prevention and eradication programs. More importantly, domestic water buffaloes are also vulnerable to many other dangerous viruses such as Bovine Viral Diarrhoea virus, Bovine Rhinotracheitis virus, and especially rotavirus group A, which is a high risk of zoonotic aetiology. Unfortunately, there is an obvious limitation in awareness of authorities and community about these viral diseases, leading to the lack of appropriate campaigns for disease control and prevention. Therefore, it is necessary and urgent to setup appropriate regulations for controlling of these diseases at both national and regional levels. However, it is either costly or time consuming to utilize of the available diagnostic tools for the purpose. These are the two key limitations, that do not satisfy requirements of disease prevention aspects and buffalo trades, preventing stakeholders to abide appropriately the present disease regulation systems, resulting in the complicated endemicity and pandemic potential of several viral diseases in Asian countries.

In an attempt to develop a new, cheap and rapid diagnostic tool, which can be used for screening to eliminate effectively buffalo including asymptomatic individuals that are carrying viruses, we found that water buffalo *(bubalus bubalis)* myxovirus resistance protein 1 (bbMx1) is the most compatible biomarker for our purpose since this type of technique has been developed successfully in human medicine for years. However, we had to deal with several difficulties since the start of this project. Firstly, the natural homeland of domestic water buffalo is Asia, which is too far away from Belgium. In addition, Belgian ruminants are free from several dangerous viral diseases such as FMD, which is currently circulating in Asia. Thus, transportation of buffalo bio-materials and specimens into Belgium is strictly regulated by national and regional regulations of control of transboundary diseases. In order to deal with the problems, we needed to conduct the molecular studies and experiments in Belgian labs, where specialized equipment and technologies are available, while the more clinical tasks including collecting bio-materials, and validation of the laboratory results were conducted in Asia. With the huge effort, we eventually succeeded in establishment of this bilateral research project,

which was conducted under the framework of a sandwich PhD thesis (between Uliège and VNUA), sponsored mainly by ARES-CCD (Académie de Recherche et d'Enseignement supérieur - Commission de la Coopération au Développement). Despite of the valuable support from ARES-CCD, a total research period of 60 months, in which only 24 months for conducting research in Belgium and 36 months for agregating samples and full-time delivering lectures at VNUA in Vietnam, is an obvious heavy constraint for this type of study. Nevertheless, the noticeable achievements of this project establish a good foundation for developing further projects.

CHAPTER I: INTRODUCTION

1. Domestic water buffalo - A valuable ruminant of Asian farmers

Domestic water buffalo (*bubalus bubalis*) is a ruminant animal originally domesticated about 3000 to 6000 years ago in Asia (El Debaky *et al.*, 2019). According to morphological and behavioral criteria, the animals are classified into two types including swamp buffalo (*Bubalus bubalis carabensis* (Fig. 1B)*;* 2n = 48) and river buffalo (*Bubalus bubalis bubalis*; 2n =50) (Fig. 1A (Hadi, 2015)), contributing about 30% and 70% to the world water buffalo population, respectively (Naveena and Kiran, 2014).

Figure 1: Domestic water buffaloes: (A) - River buffaloes; (B) – A swamp buffalo

Generally, domestic water buffaloes have some apparent characteristics in common with cattle. On the average, a new-born buffalo calf weights from 22 to 30kg, an adult male river buffalo is about 450–1000kg and an adult male swamp buffalo is about 325-450kg (Chenoweth *et al.*, 2014). The reproductive maturity age ranges from 24 to 36 months and their reproductive capacity can last up to 18 years old (Naveena and Kiran, 2014). They are high productivity livestock, well adapted to harsh environments. In addition, they can utilize low quality roughages especially agricultural crop residues and by-products to generate strong draft power, and yield high quality meat, milk and skin. Therefore, they provide significant effects on socio-economy in Asia, especially in rural areas, which have been well reviewed elsewhere (Nanda and Nakao, 2003, Borghese and Mazzi, 2005, Ranjhan, 2007, Cruz, 2010, Naveena and Kiran, 2014, El Debaky et al., 2019).

1.1. Population and distribution of domestic water buffalo in the world

According to the latest officially nominated data, by 2017 total world buffalo population reached approximately 225 millions heads, spreading over almost 50 countries over the world (Fig. 2) (FAO STAT, 2019). However, the buffalo population growth rate and distribution among countries is variable. Almost 98% of the buffaloes live in Asia while just more than 2% of those populates other continents (Fig. 3 ÷ 5) (FAO STAT, 2019).

Asia is the original and main habitat of both swamp and river buffaloes, distributed over the four main areas including the East, the South, the South East and the Central Asia. Swamp buffaloes are predominantly used for draft power and meat, accounting for about 23% to the total Asian buffalo population (Cruz, 2016). They populate mainly the East and Southeast Asian countries including: China, Thailand, Philippines, Laos, Cambodia, Vietnam, Indonesia, Malaysia and Myanmar. On the other hand, river buffaloes make up almost 80% of total Asian buffalo population. This type has greater milk productivity and is usually found in Southern Asia including India, Pakistan, Sri Lanka, Nepal and Bangladesh (Cruz, 2010).

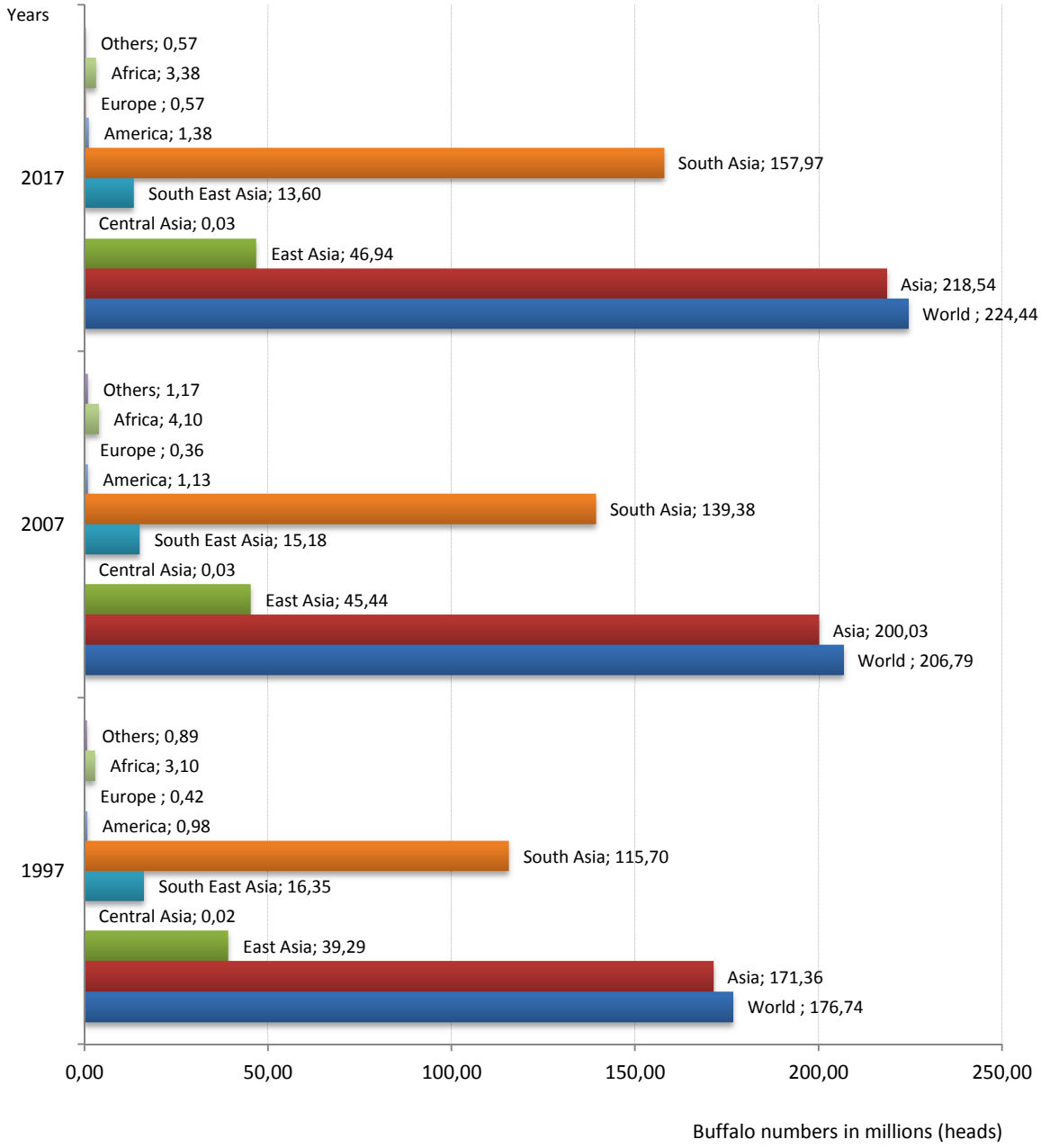

Figure 2: World buffalo population in million heads in the year 1997, 2007 and 2017

The central Asia including Kazahkstan and Tajikistan contribute the smallest buffalo numbers, just around 0,01% of the world buffalo population. China - the only one country located in the East Asia, had been always the biggest buffalo producer, contributing about 39.3, 45.5 and 47 million buffaloes in 1997, 2007 and 2017; and accounting for 22.23 %; 21.97% and 20.92% to the world buffalo population, respectively. Another top country of buffalo production is India, owning constantly around 51% of the world buffaloes (Fig. 3 ÷ 5).

Over the last 20 years, the world buffalo population has increased by 27%, from approximately 180 million heads in 1997 to almost 225 million heads in 2017. However, buffalo production countries worldwide experienced both increase and decline in their buffalo heads (Fig. 6). Majority of the said countries obtained a linear increase in their buffalo numbers. Kazakhtan increased its buffalo herds by 3,82%, from 10.000 heads in 1997 to 10.382 heads in 2017. Interestingly, Pakistan and Italy rocketed their buffalo numbers by 80,92 % and 167,19%, respectively. On the other hand, some countries struggled with a sharp drop in their number of buffaloes. Approximately 78% (7 out of 9 countries) of South East Asian countries including Thailand, Indonesia, Malaysia, Vietnam, Cambodia, Philippines and Laos, decreased buffalo numbers by 65,22%; 54,47%; 20,88%; 15,35%; 14,46%; 2,91% and 2,84%, respectively, causing the total buffalo number of this areas in 2017 dropping by 16,81% compared to 1997.

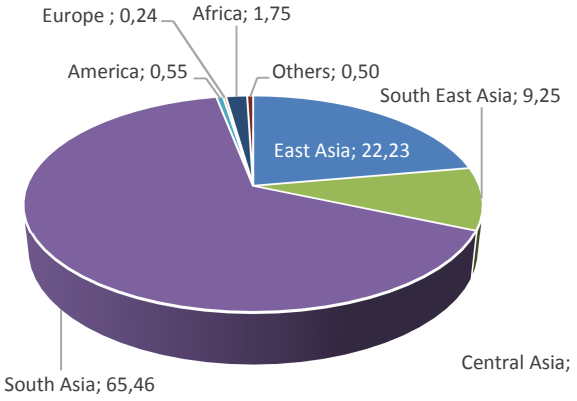

Figure 3: World water buffalo distribution (%) in 1997

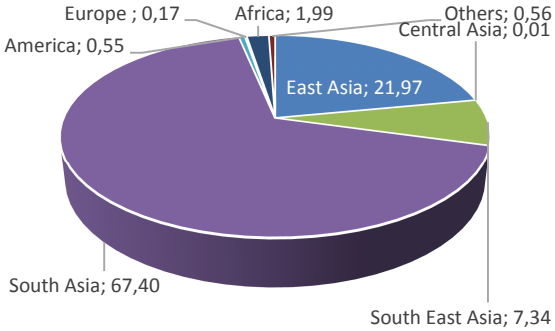

Figure 5: World water buffalo distribution (%) in 2007

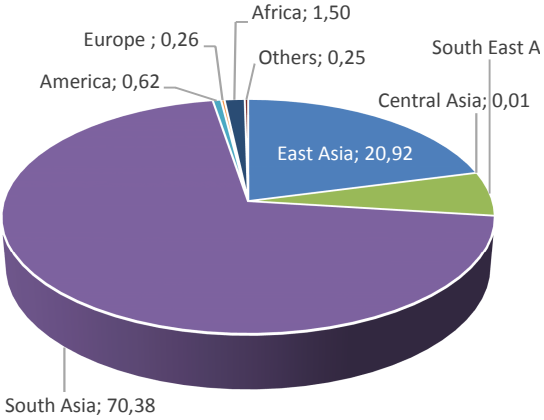

Figure 4: World water buffalo distribution (%) in 2017

Regardless to the causes of the decline in buffalo numbers in some countries; the gap among the positive vs. negative buffalo growth rate and increasing demand from market have created high pressure on transportation of buffalo and buffalo-derived products in both domestic and oversea levels. This is one of the intrinsic forces, forming Asia especially East and South East Asia to be the most dynamic buffalo market, which contributes significant impact in economic development in the areas, however it also challenged infectious disease control for not only buffaloes but also for other livestock.

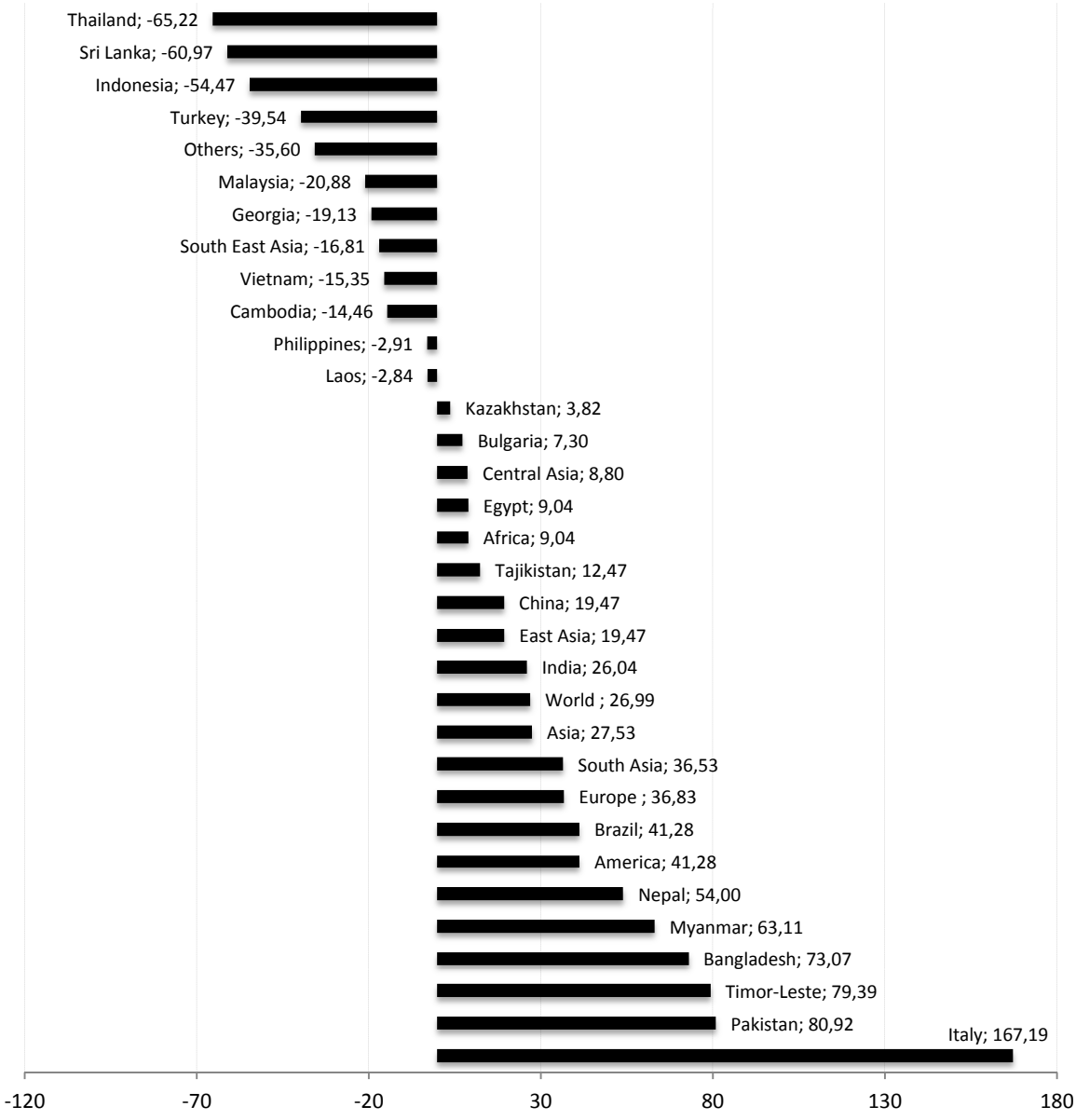

Figure 6: World buffalo growth rate (%) of major countries over the last 20 - year period, from 1997 to 2017

1.2. Socio-economic roles of domestic water buffalo in Asian countries

Domestic water buffaloes have impacted significantly socio-economic aspects in many Asian countries, especially in rural areas. They have been used not only as the effective draft power source for agriculture cultivation and transportation but also for their high productivity of meat and milk. Therefore, the domestic water buffaloes are always considered as the most value property of many farmer families in Asian countries. It is predicted that expanding production of meat and milk-oriented buffaloes is one compatible strategy to adapt to the vast increasing in meat, milk and grain consumption due to the explosion of Asian and global population.

Figure 8: A ploughing buffalo on a ladder field in highland area in northern Vietnam.

Figure 7: Leonora – a dairy buffalo breed.

In Asia, domestic water buffaloes provide major draft power for agricultural cultivation (Fig. 7) (Tri, 2019). An adult buffalo normally can continuously pull a load of about 2.0 tons or even up to six times of their own bodyweight for 2–3h. On average, they can work up to the age of 11 years old (Nanda and Nakao, 2003) contributing from 30% to almost 80% of farm power depending cultivation soil conditions and mechanization levels (Warriach *et al.*, 2015). A draft buffalo usually works for around 5 h a day and up to 146 days per year in Thailand, meanwhile it is about 109 days per year in Vietnam (Nanda and Nakao, 2003). In the past 30 years, before mechanization was applied in agriculture, each farmer family usually owned from 1 to 3 buffaloes to exploit for draft power and manure. Nowadays, in the large, flat and dry field soils, buffalo draft power has been replaced significantly by machines and become less important in soil preparation for agriculture cultivation. However, buffaloes are still the most effective draft source in the waterlogged (muddy) conditions such as water rice paddies in Hong river delta and Mekong river

delta areas or in the mountainous and remote areas of Vietnam for instance, where utilization of machine is still impossible or with a low level.

Water buffalo is also a high-quality milk source for human daily consumption in many Asian countries (Fig. 8) (Aselmo, 2017). In comparison to cow milk, buffalo milk contains higher nutritional components including: fat, lactose, protein, total solids, and vitamins and minerals such as calcium, magnesium, and inorganic phosphate, therefore buffalo milk and milk products are preferred in many Asian countries (Wanapat and Kang, 2013). In total annual milk production worldwide, water buffaloes milk accounts for more than 13.2%, consequently water buffaloes become the second most important dairy animals after dairy cows (Han *et al.*, 2012). Milk productivity varies, depending on buffalo types. River buffaloes are milk-oriented and have highest productivity, each buffalo can produce between 1000 and 3000kg annually. On the other hand, swamp buffaloes are originally used for draft power and meat (El Debaky *et al.*, 2019) therefore milk productivity is much lower, just about 600kg annually. Domestic water buffaloes contribute about 100 million tons of milk annually; representing for about 13,2% to the total world milk productivity (Correddu *et al.*, 2017).

In addition, water buffaloes are also high meat producing animals (Fig. 9). Their meat is specially preferred in Asia and has become more popular in several countries. On average, a buffalo calf can gain body weight from 0.66 to 0.85kg per day under free grazing conditions or up to 0.9 to 1kg under intensive feeding with balanced diet (Naveena and Kiran, 2014). The average ratio of carcass to live body weight is around 52%. Buffalo meat is red colour, high protein, desirable texture, low fat and low connective tissue; especially it faces no religious prohibition of consumption (Naveena and Kiran, 2014, Kandeepan *et al.*, 2013). In 2012, only top five countries including India, China, Pakistan, Nepal and Egypt already contributed about 198.88 million tons of water buffalo meat to approximately 50 countries around the world, contributing significantly to the economy of the host country (Naveena and Kiran, 2014).

Figure 9: Swamp buffaloes loaded at Cho U market (Nghe An, Vietnam) for slaughterhouse.

In culture and religion, buffaloes also play important roles in spirits of Asian communities. The animals are used in several culture festivals such as buffalo fighting festivals in the northern and buffalo sacrifice festival of Ba Na ethnic people in Tay Nguyen, Vietnam. In some other communities such as northern Laos, buffaloes are considered as human and they are usually used as sacrifices for ill persons with the hope that they can help to recover the patient's health and save his life (Sprenger,

2005). Even if these events are supposed to be violent and should be stopped, their spirits still play certain roles in the local communities and it is not easy to eliminate.

Furthermore, buffaloes also provide an excellent manure source for crop and fuel production. On average, each adult buffalo produces about 4-6 tons of wet manure, annually. In Asian countries, manure is frequently used from about 51 kg (Vietnam) up to 21.7 tons/ha (China) to spread on crops once or twice per year. Furthermore, buffalo dung is also an important material for production of biogas, providing crucial energy source for heat and electricity generation. It is estimated that buffalo manure makes up approximately 40% of the total value of the animal. In Pakistan for example, buffalo manure contributes about 920 million rupees per year (Nanda and Nakao, 2003).

However, there has been a gradually change in orientations of using domestic water buffalo in Asian countries due to the rapid mechanization in agriculture, urbanization and explosion of Asian population. Draft buffaloes have been gradually less important in agriculture cultivation and transportation in rural areas. In Vietnam for instance, the utilization of machines on agriculture cultivation has yielded greater productivity since 1990s, compared to using buffalo draft power. In addition, rapid urbanization and industrialization also decreased agricultural land areas where draft buffaloes are used to work for centuries. Consequently, domestic water buffaloes, especially draft-oriented type have been losing rapidly their role in providing draft power. On the other hand, the explosion of human population and improvement of life quality in the areas result in increasing demand of daily red meat and milk consumption. Taken all together, many draft-oriented domestic water buffaloes have been slaughtered for meat and other buffalo-derived products. This is one of the most important reasons why the water buffalo population in Southeast Asia has been constantly dropping over the last 20 years; meanwhile the demand of buffalo meat and milk still increases markedly. Therefore, development of meat and milk-oriented domestic water buffaloes intensively in larger scales is a necessary trend and compatible strategy for both economic and agricultural aspects (Naveena and Kiran, 2014).

Asian buffalo trade, risk of trans-boundary diseases and potential measures

Domestic water buffaloes have been recognised increasingly important in Asia, especially in East and Southeast Asia, where the buffalo population has decreased gradually over the last 20 years. In addition, the explosion in human population in the area also results in a rapid increase in demand of buffalo meat, milk, and other derivable products such as cheese and yoghurt. Therefore, trade of livestock and buffaloes in particular have become increasingly vibrant, contributing obvious effect on the economic development. However, it also induces great challenges to veterinarian authorities for controlling trans-boundary viral diseases.

1.3. Asian buffalo trade: matrix movement pathways and trans-boundary diseases issues

Asia contains massive network of large ruminant markets with complicated movement pathways for exchanging thousands of livestock including domestic water buffaloes daily. China and Vietnam are the biggest buffalo markets, driving some significant changes in animal movement patterns throughout the region. Annually, millions of domestic water buffaloes have been traded from several livestock markets throughout the surrounding countries.

The traded buffaloes vary in body sizes, ages and origins. The animals are usually collected firstly from several farmer families, conducted by small-scale local traders before merging in assembly markets for selling to intermediate traders. Subsequently, the purchased animals are transported over huge distances and sometimes cross many borders to arrive in China and Vietnam, where they are continuously delivered to further destinations (Fig. 10 & 11). Majority of the traded buffaloes is slaughtered for meat either soon at slaughterhouses or after fattening period in feedlots, while a minor proportion is used for breeding purpose.

Figure 10: Cho U market, located in Nghe An province in the middle of Vietnam.

Notes: This is one of the biggest cattle markets, opening 6 times monthly and exchanging about 10,000 animals per market session).

According to Smith *et al.*, (2015) it was estimated that, from 2013 China imported around 1.25 to 1.5 million of alive buffaloes per annum, in which more than 75% originated from several collection markets in Myanmar, and about 20% was imported from Vietnam and the rest from other countries such as Laos and Thailand.

On the other hand, Myanmar, Thailand, Australia and Laos have been the main buffalo exporters with about 720 thousands to around 1 million heads annually to Vietnam, which is a big destination and also an important intermediate transit port to China (Smith *et al.*, 2015).

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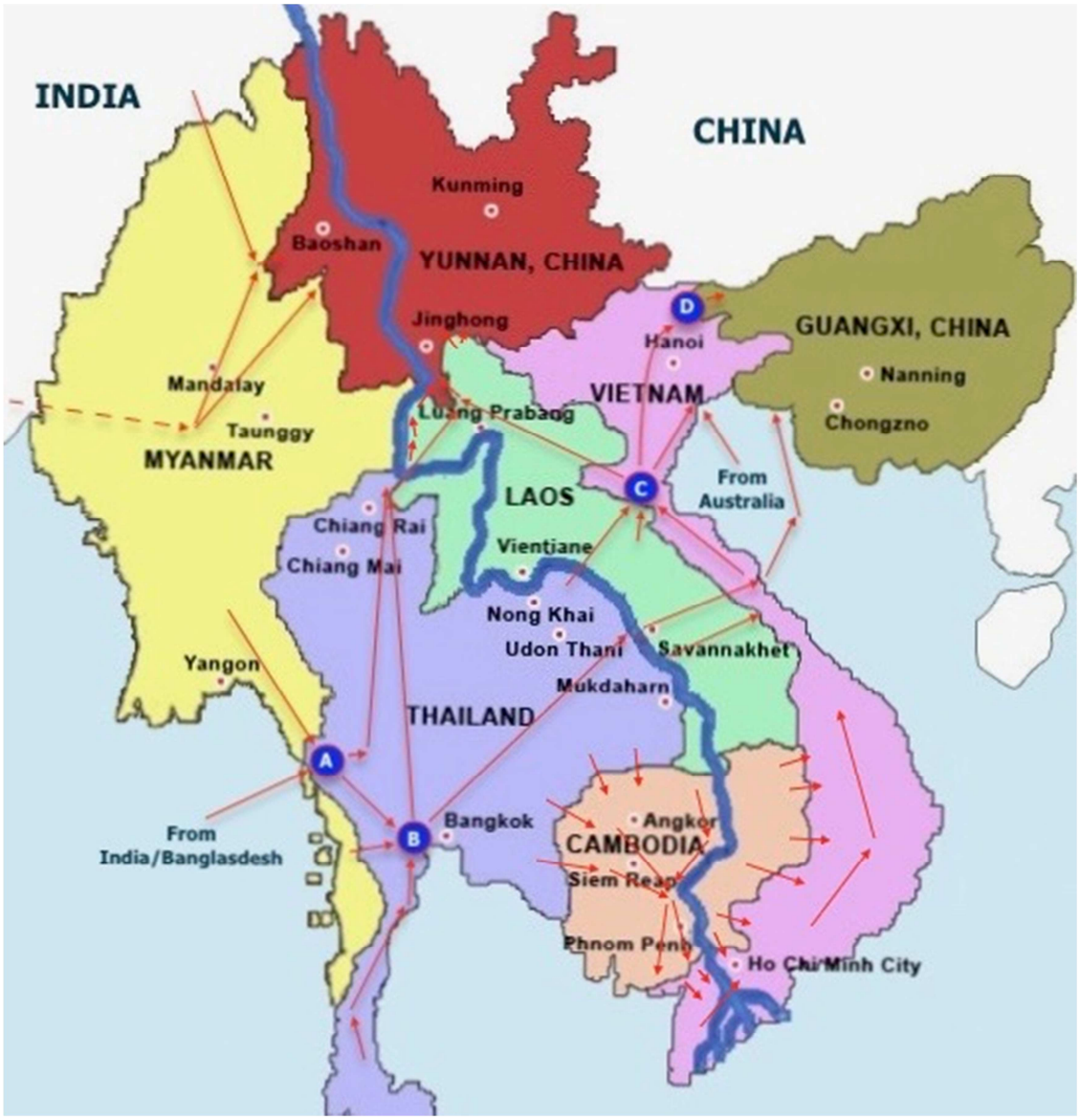

Figure 11: The main pathways of livestock movement in Greater Mekong Sub-Region countries.

(Sources: adapted from Kerr et al., 2012; Smith at al., 2015 and GMS Map, the Mekong Tourism Coordinating Office)

2 Notes: The map illustrates the main collection areas along the movement pathways destined for China and
3 Vietnam; and estimated numbers of large ruminants passing through those areas each year. The continuous
4 red arrows indicate the movement pathways crossing borders. The dot red arrow indicates the unclear
5 pathways. (A): Photong market located in Tak province, Thailand. Trading around 520 thousand heads per
6 annum (about 5,000 heads per market session at every 4-days interval). (B): Fattening plot, located nearby
7 Bangkok, Thailand. (C): U market, located in Nghe An province, Vietnam. Trading around 720 thousand heads
8 annually (about 10,000 heads per market session at every 5-days interval). (D): Hung Quoc market, located in
9 Cao Bang province, Vietnam. Trading around 150 thousand heads annually (about 2,000 heads per market
10 session at every 5-days interval).

It is important to highlight that a large proportion of the traded buffaloes follow unofficial pathways from multi-origins and without appropriate health inspection (Fig. 12) (Smith *et al.*, 2015). Many of those are purchased from several farmer families in different locations including remote areas, where the vaccination programs are usually limited or even with endemic circulation (Sieng *et al.*, 2012). Thus, it is possible that a certain number of the animals were asymptomatic pathogen carriers. They could spread pathogens to not only others animals in the same market session and transportation vehicles but also along the movement pathways and at the destinations, threatening the biosafety of the whole region.

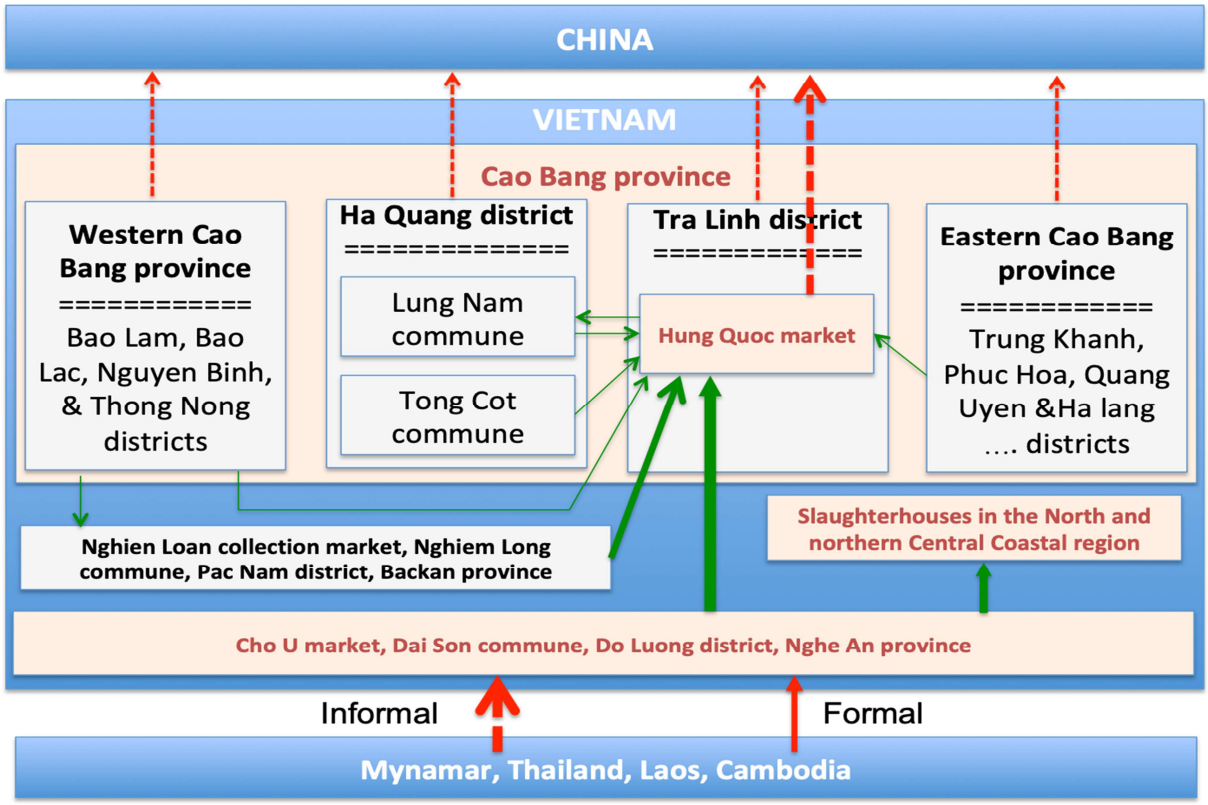

Figure 12: Schematic illustration of main movement pathways of buffalo and cattle trade via Vietnam destining to China.

Notes: The red arrows indicate the direction pathways crossing borders. The green arrows indicate the direction of domestic movement. The continuous arrows indicate the official movements whereas the dot once indicates unofficial movements (Adapted from Smith et al., 2015).

It is clear that risks of spreading trans-boundary diseases are in proportion to numbers of traded animals circumventing the disease inspection systems, the scale of the assembly markets and the number of traded animals' origins. Therefore, regardless to the significant contribution of buffalo trade to the Asian economic development, movements of the livestock are an important factor for widespread of several animal trans-boundary diseases throughout the region. This could be the main underlying mechanism of the several ongoing infectious diseases in livestock including FMD in cattle in Asian region.

1.4. Current trans-boundary diseases control systems: drawbacks and need of innovation

In order to prevent animal trans-boundary diseases, each Asian country has its own regulation system for health management of livestock movements at both domestic and overseas levels.

In Vietnam, domestic movements of livestock among provinces is approved officially in accordance with articles 38 and 48, chapter 3 of the Vietnam's law on veterinary medicine, issued in 2015. The traders must obtain all of the following documents/conditions (Hung, 2015):

(1) Certificates confirming that registered animals have obtained enough immunological protection levels against given number of infectious diseases depending on certain departures and destinations;

(2) Pass the clinical examination conducted by veterinary authorities upon the departure;

(3) Animal health Certification for transportation, issued by provincial department of animal health. This process usually takes about 1 to 5 working days depending on the origins of the animals.

On the other hand, import of livestock is approved officially in accordance with articles 46, 47 and 48, chapter 3, of the Vietnam's law on veterinary medicine, issued in 2015. Traders must obtain the following documents/conditions (Hung, 2015):

(1) Animal health Certification for export, issued by national department of animal health of the export country in accordance with requirement of Vietnam.

(2) Animal Quarantine Certification. At the border of Vietnam, the animals must be kept in the quarantine areas for around two weeks or up to 45 days for disease inspection.

Technologically, the regulation seems to be adequate for disease control, however in term of economy, the long duration for processing applications certainly creates numbers of crucial limitations. Firstly, it prolongs significantly the transportation time. Consequently, it deters the trade pace and possibly interferes traders to obtain the optimal selling chance at the destination. Secondly, it increases price due to the additional cost for taking care of the animals in the quarantine areas. Thirdly, keeping livestock in quarantine areas also increase risk for cross infections among quarantined animals if any asymptomatic animals are accidently included in the herd.

In addition, a high proportion of borders between Asian countries is located along the mountainous and remote areas, providing good conditions for unofficial movements of livestock and their products crossing the borders. Therefore, the majority of traders circumvents the regulation systems at the border checkpoints by employing local people living along the border to walk illegally their cattle and buffaloes in small groups to cross border. Then, they are transported to assembly markets under the mask of local farmers' property. Thus, instead of applying regulation for overseas transportation at the border, they will be approved in accordance with regulation for domestic

transportation in the markets, which is much less strict and faster than the overseas level. This trick helps many livestock traders to import the animals easily by circumventing quarantine step (Sieng *et al.*, 2012). Thus, they can save significant costs and time due to the fact that the circumvented animals can receive health certificate just by clinical examination, which only can identify active or recent infections, meanwhile it is impossible to detect disease-incubating or carrying virus post-infection animals. Consequently, such kind of the unregulated movements of cattle and other livestock has been threatening biosecurity over the whole Asian region (Sieng *et al.*, 2012). A good evidence supporting for this conclusion is that many Asian island countries such as Indonesia, Philippines, and the states including Sabah and Sarawak – Malaysia, where such kind of unofficial movements of livestock and animal products do not have chance to exist, are free from FMD – the most economically important trans-boundary disease – even without vaccination (Fig. 13) (Blacksell *et al.*, 2019).

In Southeast Asia, many regional campaigns have been established for controlling animal trans-boundary diseases. For instance, the South-East Asia Foot-and-Mouth Disease (SEAFMD) program was established in 1997, and then it was modified to The South-East Asia and China Foot-and-Mouth Disease (SEACFMD) when China jointed in 2010 (Abila *et al.*, 2012). Another regional system is The Malaysia–Thailand–Myanmar (Burma) (MTM), established in 2003. These programs aim to establish a FMD-free region by strengthening cooperation among country members by improving the disease communication, surveillances and standardized regulation systems. The campaigns have contributed to the mitigation of FMD in Southeast Asia and China.

However, despite of the consistent effort of each country members, the successful control of animal trans-boundary disease within Southeast Asia has been deterred by several factors including unregulated 'informal/unofficial' movements of livestock and their products, difficulties implementing vaccination programs, complicated mutations of pathogens, low-level technical capacity in early detection of infected animals, limited farmer knowledge on infectious diseases and biosecurity, failure of timely outbreak reporting and response and limitations in national and international trans-boundary diseases control programs (Blacksell *et al.*, 2019). These limitations led to either failure of timely detection of asymptomatic animals prior to the movement or dissatisfying livestock traders and markets in term of trade pace and additional cost to their stock. Consequently, they deter livestock trade stakeholders including farmers, traders and other authorities to abide the regulations, resulting in the unsuccessful control systems of animal trans-boundary traffic in the region.

1.5. Common buffalo trans-boundary diseases in Southeast Asia: status and control measures

According to the World Organization for Animal Health (OIE) 2019, trans-boundary diseases on livestock in Asia have been circulating complicatedly. Millions of livestock have been affected with several dangerous infectious diseases including Highly Pathogenic Avian Influenza (HPAI) and Newcastle disease (ND) in poultry, Porcine reproductive & respiratory syndrome (PRRS), FMD, and

African Swine Fever (ASF) in swine, Peste des petits ruminants (PPR) and FMD in cattle (OIE, 2019). However, the scope of this review is focusing on epidemiology of viral diseases in domestic buffaloes and cattle.

1.5.1. Foot-and-Mouth Disease

Foot and Mouth Disease is one of the most economically serious endemics, attracting predominantly attention from both authorities and farmers in Asia for approximately 150 years. Foot-and-Mouth Disease virus (FMDV) is a highly antigenetically variable and contagious pathogen of cloven-hoofed animals. The virus belongs to *Aphthovirus genus* within the *Picornaviridae* family, and exists as more than 60 sub-serotypes derived from seven distinct serotypes including: O, A, C, Asia 1, Southern African Territories 1 (SAT 1), SAT 2 and SAT 3, in which serotypes O, A and Asia 1 have been circulating frequently in Southeast Asia (Abdul-Hamid *et al.*, 2011, Bui Ba Bong, 2006). Noticeably, these serotypes cause the same symptoms and lesions. However, they do not produce cross immunization, meaning that animals vaccinated with serotype A are not protected against the other serotypes. This makes the disease more costly and difficult to eradicate.

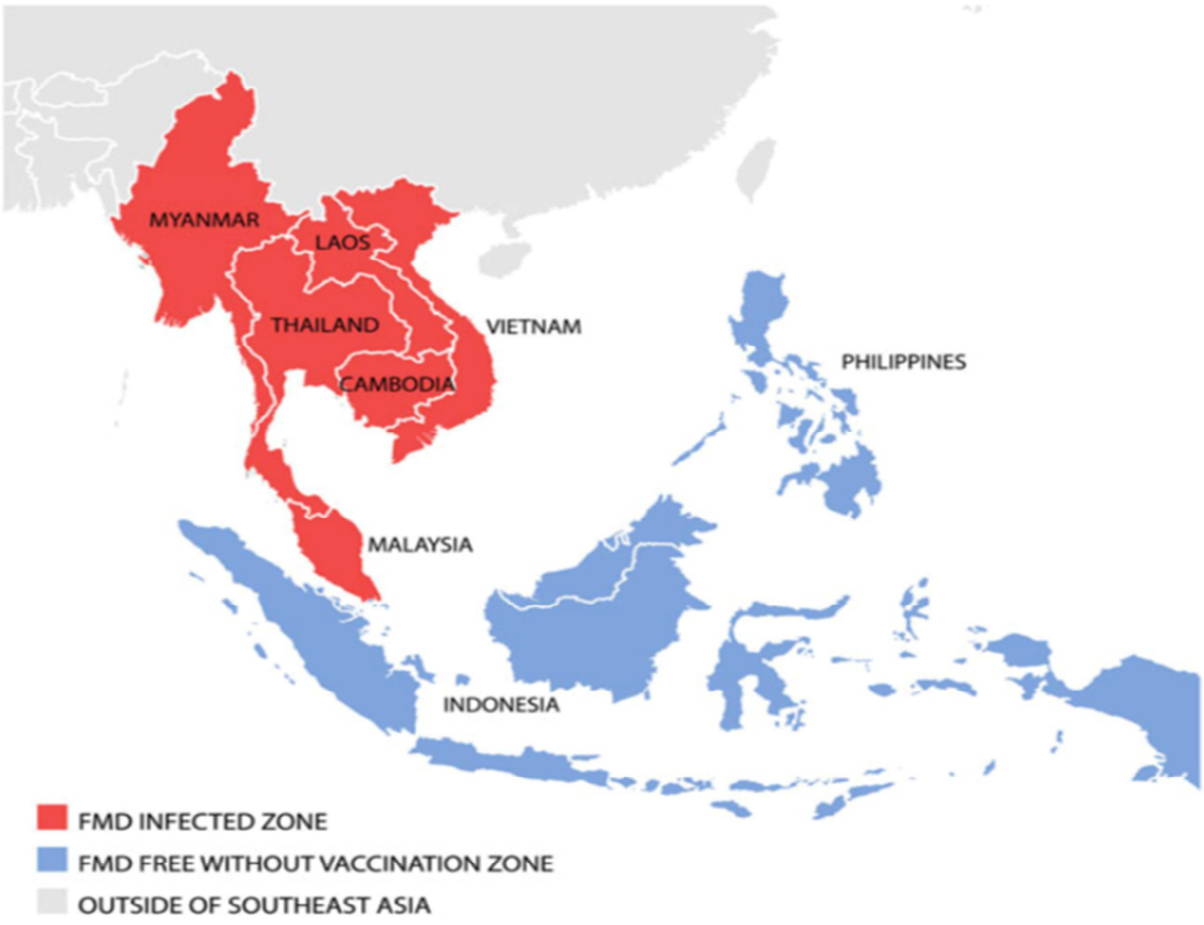

Figure 13: Distribution of foot-and-mouth disease in Southeast Asia

Over the period from 2007 to 2017, FMDV has circulated variously in In Southeast Asia, first FMD outbreaks were observed approximately 150 years ago in Indonesia, Malaysia and the Philippines. Then, the endemic has spread to over all of the Southeast Asian members and China including Myanmar, Laos, Thailand, Cambodia, Vietnam, Malaysia, Indonesia and the Philippines. Interestingly, after a long consistent effort by individual nations and regional cooperation, island countries including the Philippines, Indonesia, and Sabah and Sarawak – the two island states of Malaysia - have eradicated FMD. Meanwhile, the other Southeast Asian members still have been struggling with the endemic (Fig. 13) (Blacksell *et al.*, 2019) & (Fig. 14) (Karanvir and Ronello, 2016).

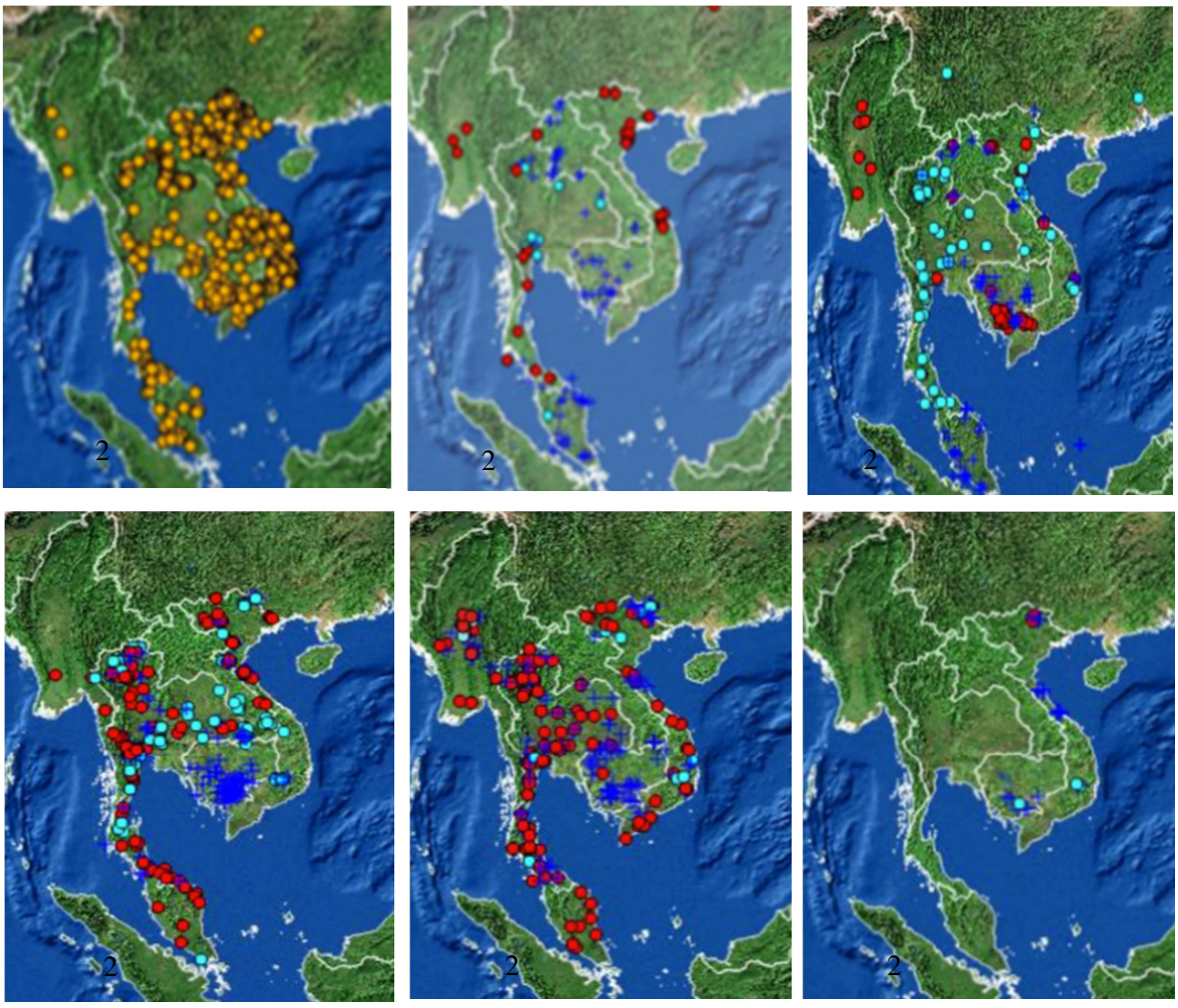

Figure 14: FMD map in Southeast Asia from 2011 to 2016

Notes: The orange dot indicates unknown FMDV serotype, the red dot indicates FMDV

Southeast Asian countries including Laos, Cambodia, Thailand, Vietnam, Cambodia and Myanmar (Fig. 15) (Blacksell et al., 2019). In overall, the number of FMD outbreaks varied in each year and nation, and it has decreased gradually except for the unusual peak in the year 2011. The average annual outbreak number is about 493. Over whole period, FMD has been incriminated in 4,926 outbreaks, in which virus serotypes O, A and Asian 1 were found to be the cause of 1,416,361 and 6 outbreaks, respectively. Meanwhile, 3,143 of the outbreaks were unclassified for virus serotype, accounting for ~73% of the total outbreaks. This is a big gap leading to difficulties in selection of the compatible vaccine for disease prevention in the outbreak's areas since distinct FMDV serotypes do not produce cross immunization. Therefore, vaccination program will be ineffective if they do not contain virus serotypes which are compatible with the circulating serotype in the relevant areas.

As can be seen in Fig. 15 and Table 1 (Blacksell *et al.*, 2019), from 2010 to 2018, FMDV circulating in Southeast Asia belongs to serotype O, A and Asian 1 under a total of 7 lineages in which 5 lineages were derived from serotype O including: Middle East – South Asia/PanAsia – [ME-SA. (PA)], Middle East – South Asia/India-2001d – [ME-SA (Ind-2001d)]; Middle East – South Asia/India-2001e – [ME-SA (Ind-2001e)]; South East Asia/Myanmar-98 - [SEA (Mya-98)]; and Cathay – CHY. On the other hand, just only lineage ASIA (Sea-97), and ASIA and ASIA (G-VIII) were found to be derived from serotype A and Asian, respectively (Table 1) (Blacksell *et al.*, 2019).

The locations of these lineages demonstrated their movement pattern throughout the region. Vietnam, Thailand and Myanmar, which are the most dynamic livestock markets have the highest diversities of FMDV lineages. Meanwhile it was relatively stable in Malaysia, Cambodia and Laos, where the livestock movements were much less frequent. In 2007, the O/ME-SA/PanAsia lineage was found in Laos, Vietnam and Cambodia. One year later, it spread to Thailand, and circulated constantly within the four countries until the end of 2017, followed by the introduction of two new linages including O/ME-SA/Ind-2001e and O/ME-SA/Ind-2001d in the region. Similarly, Cathay linage (O/CHY) was first introduced into Southeast Asian region via Thailand in 2012, and then it disappeared absolutely from this country before reappearing in Vietnam in 2016 and 2017.

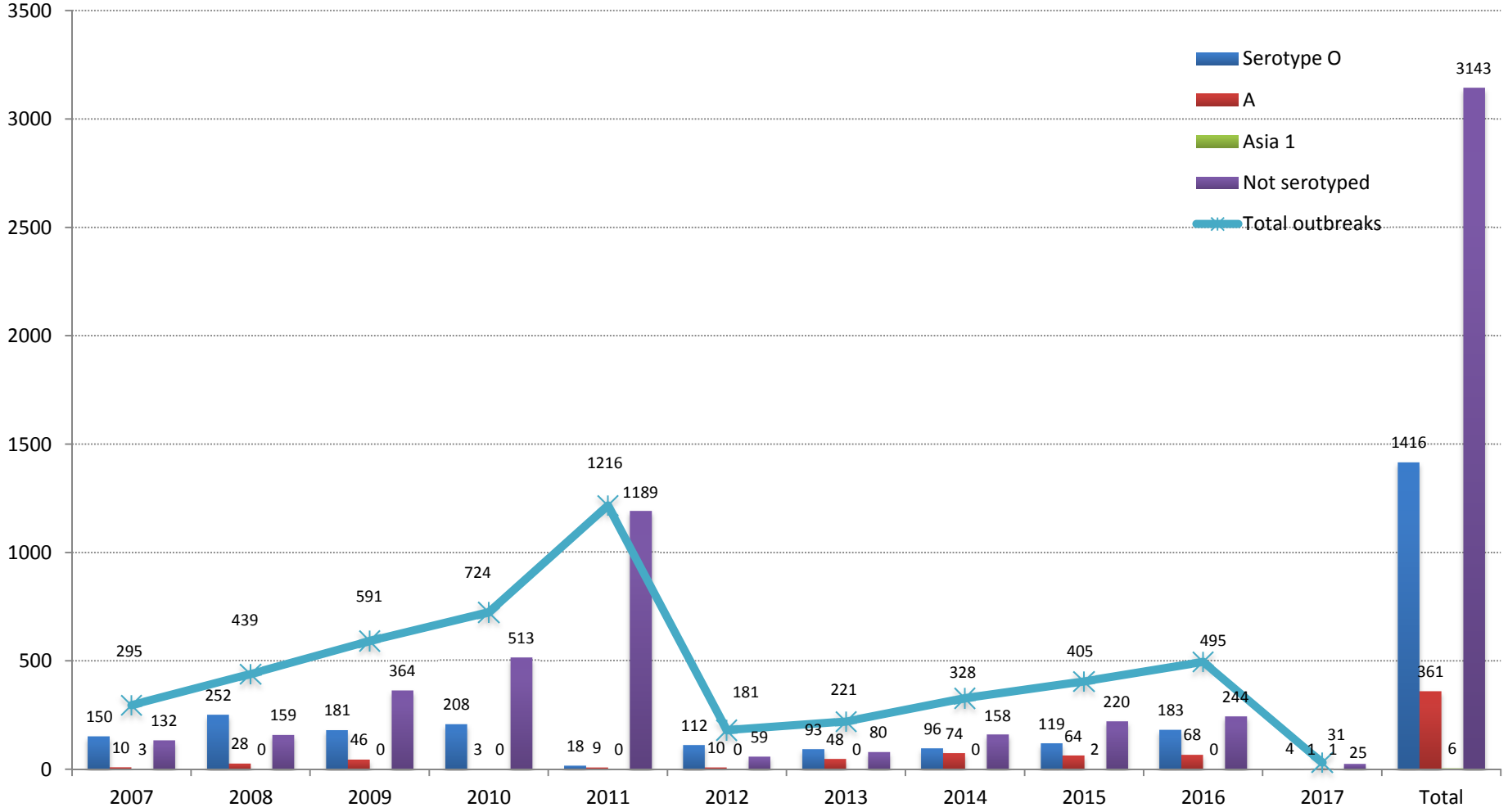

Figure 15: Numbers of outbreaks with specific serotypes of FMDV in Southeast Asia from 2007 to 201

Table 1: Summary of FMD serotypes, topotypes and lineages circulating in Southeast Asia from 2010-2018

Countries	Serotype	2010	2011	2012	2013	2014	2015	2016	2017	2018
Myanmar	O	SEA (Mya-98)					SEA (Mya-98)	ME-SA (Ind-2001d)	ME-SA (Ind2001e), ME-SA (Ind-2001d), SEA (Mya-98)	
	A	ASIA					ASIA (Sea-97)			
	Asia 1								ASIA (G-VIII)	
Laos	O	ME-SA (PA), SEA (Mya-98)	ME-SA (PA)	ME-SA (PA)	ME-SA (PA)		ME-SA (Inde-2001d)	ME-SA (PA)	ME-SA (PA)	
	A	ASIA				ASIA (Sea-97)	ASIA (Sea-97)			
Thailand	O	SEA (Mya-98)	ME-SA (PA), SEA (Mya-98)	CHY, ME-SA (PA), SEA (Mya-98)	SEA (Mya-98)	SEA (Mya-98)	ME-SA (PA), SEA (Mya-98)	ME-SA (PA), SEA (Mya-98)	ME-SA (Ind-2001e), ME-SA (PA), SEA (Mya-98)	ME-SA (Ind-2001d), ME-SA (PA)
	A	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)
Vietnam	O	ME-SA (PA), SEA (Mya-98)	ME-SA (PA), SEA (Mya-98)	ME-SA (PA)	ME-SA (PA)	ME-SA (PA), SEA (Mya-98)	ME-SA (Ind-2001d) ME-SA (PA), SEA (Mya-98)	CHY, ME-SA (PA), SEA (Mya-98)	CHY, ME-SA (Ind-2001d), ME-SA (Ind-2001e), ME-SA (PA), SEA (Mya-98)	
	A	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	
Cambodia	O	ME-SA (PA)	ME-SA (PA)	ME-SA (PA)	ME-SA (PA)		ME-SA (PA)		ME-SA (PA)	
	A						ASIA (Sea-97)		ASIA (Sea-97)	
Malaysia	O	SEA (Mya-98)	SEA (Mya-98)	SEA (Mya-98)	SEA (Mya-98)	SEA (Mya-98)	SEA (Mya-98)	SEA (Mya-98)	ME-SA (Ind-2001e)	ME-SA (Ind-2001e)
	A		ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)	ASIA (Sea-97)				

Abbreviations: PA- PanAsia; ME-SA- Middle East–South Asia; SEA - Southeast Asia; Mya - Myanmar. Ind - India.

In Vietnam, the first FMD outbreak was reported in Nha Trang - the central region - in 1898, and then it has spread throughout the country and infected hundred thousands cloven-hoofed animals. Interestingly, the disease was absent in northern Vietnam for 32 years, from 1960 to 1992, when the border was closed without international commercial exchanges.

Figure 16: Sampling of a FMD infected swamp buffalo in Lai Chau province of Vietnam in 2017

However, Vietnam government has started the open policy for international cooperation and trading since 1991. Then, accompanying the increase in oversea exchanges, FMD spread throughout the country (Fig. 16). Over the last 20 years, from 1999 to 2018, the epidemic infected 719,350 cattle (and almost 1 million pigs, this data was not displayed) in 7,804 outbreaks throughout the country. In the year 2000, FMD reached a peak by spreading to 1,708 communes (endemics) throughout the country, infecting 351,284 ruminants (buffaloes & cows) (Fig. 17) (Vietnam Department of Animal Health, 2011). Among which, infected buffaloes contributed predominantly with an overall of 70.94% to the total infected cattle over the period from 2011 to 2018 (Fig. 18). Unfortunately, these detailed data were not available for the previous period. In addition, hundred thousands pigs were also infected with FMDV, which were eliminated in accordance with Vietnam law on veterinary medicine. FMD has damaged seriously animal production and the economic development of the country.

In order to control and eradicate the pandemic, several national and regional programs have been established for FMD control. For this considerable effort, during the last 10 years, Vietnam government has spent more than 6 millions USD per annum for purchasing vaccines, strengthening veterinary personnel and establish of FMD control and surveillance systems (Vietnam Department of Animal Health, 2011). In addition, Vietnam government also has coordinated with OIE, FAO and some other regional FMD programs such as South-East Asia Foot and Mouth Disease (SEAFMD), formally established in 1997 by Cambodia, Lao PDR, Malaysia, Myanmar, the Philippines, Thailand and Vietnam. Then, in 2010, with the participation of China, SEAFMD was renamed “South-East Asia and China Foot and Mouth Disease” (SEACFMD).

The programs have contributed to significantly mitigate of FMD in Vietnam as well as in other member countries. In Vietnam, number of cattle and communes that were infected by the FMDV decreased from 351,284 and 1,708 in the year 2000 to just 733 and 20 in the year 2018, respectively (Fig. from 17 to 19). This was a great achievement; however, it will take much longer time and effort to eradicate absolutely the disease from the region.

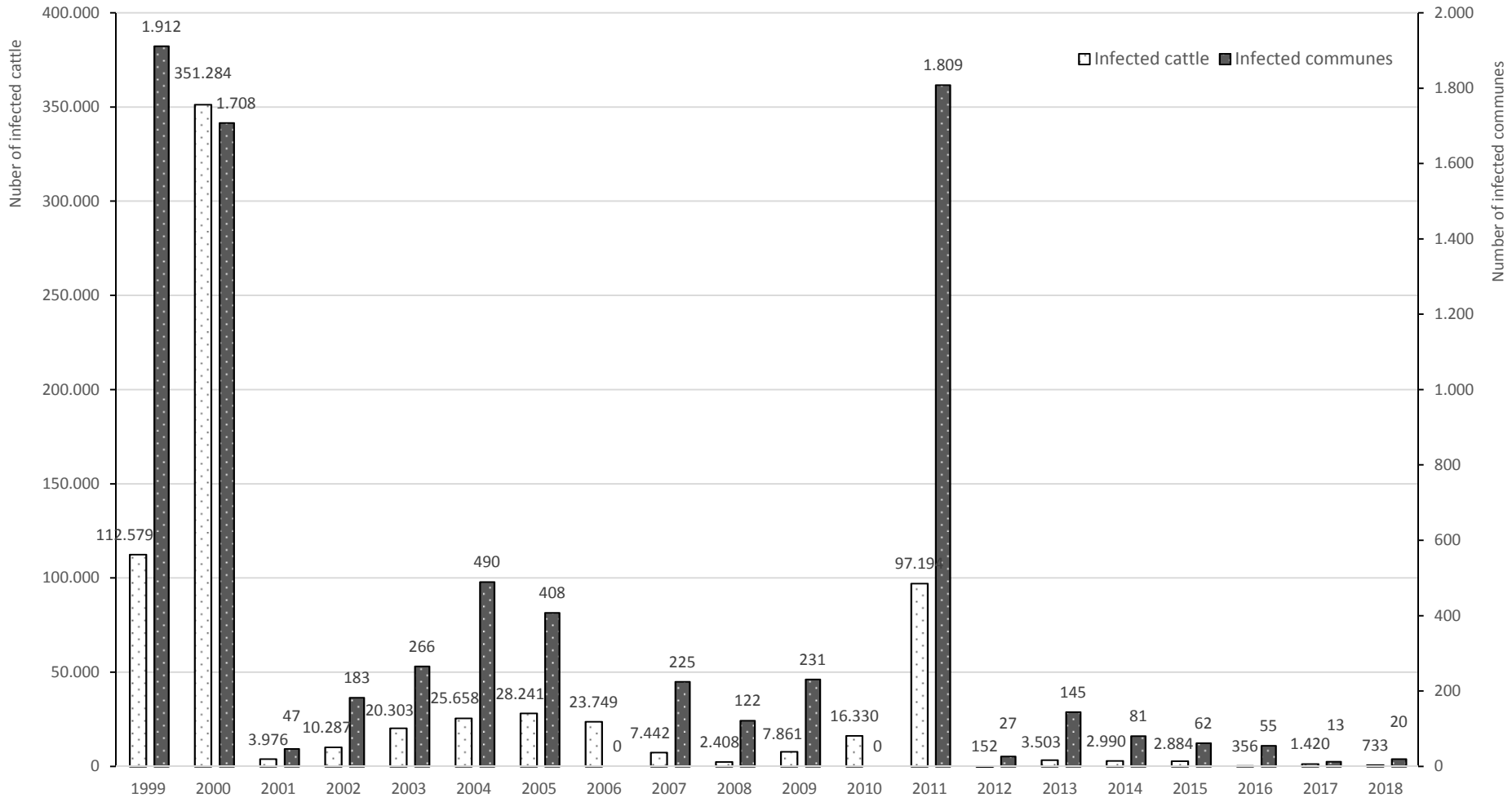

Figure 17: Number of cattle and communes infected with FMD in Vietnam from 1999 to 2018

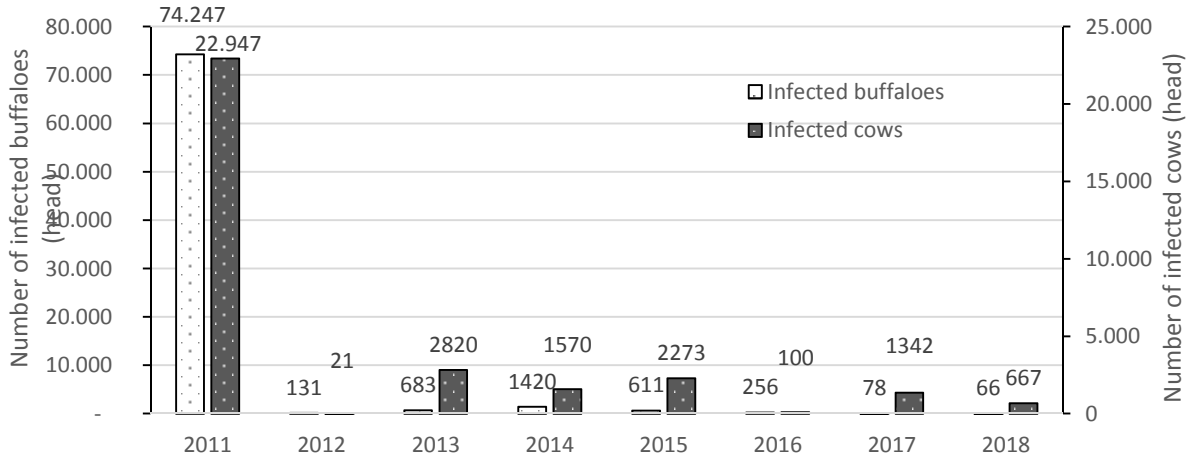

Figure 18: Number of buffaloes and cows infected with FMDV in Vietnam from 2011 to 2018

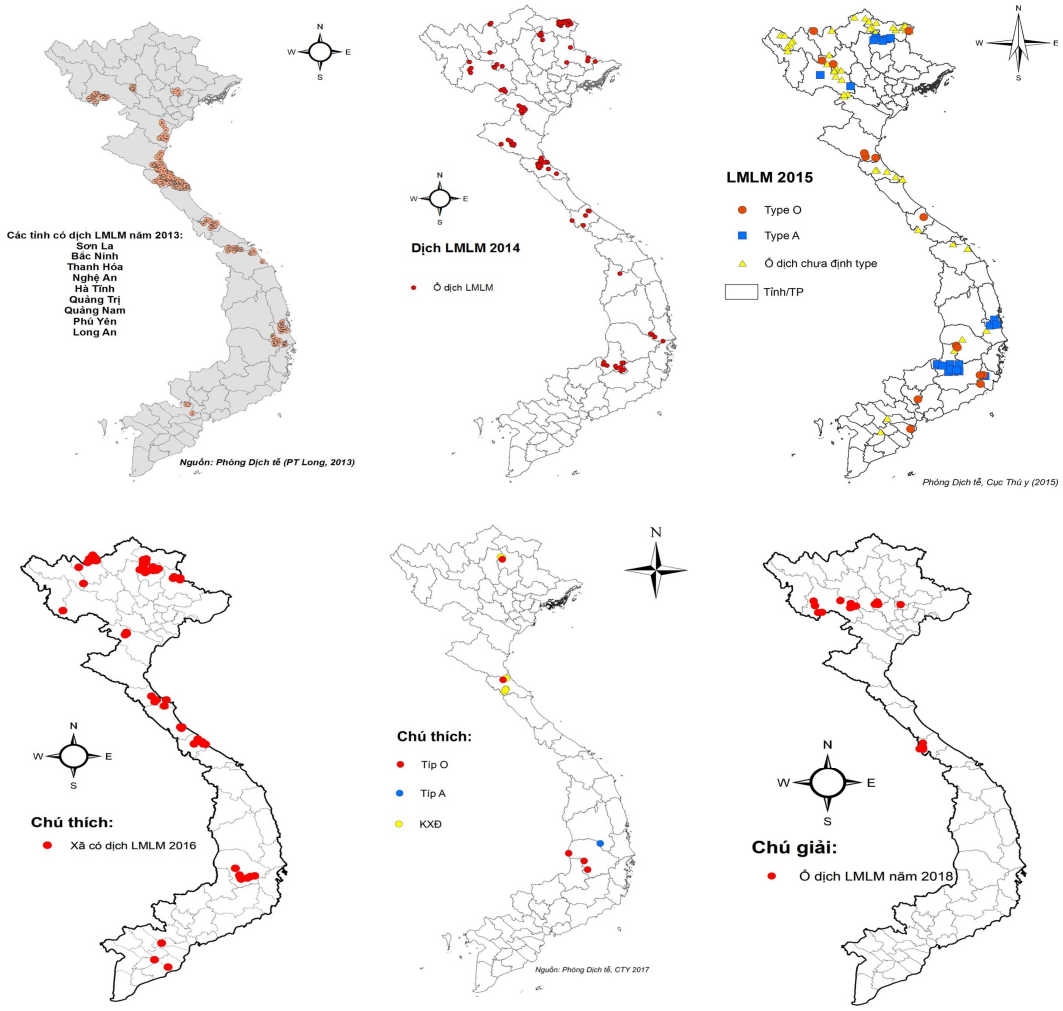

Figure 19: Epidemiological map of FMD in Vietnam from 2013 to 2018.

(Source: Vietnam National department of Animal Health, 2019)

1.5.2. Other animal viral diseases

Recent studies have shown that, besides FMD, domestic water buffaloes are also vulnerable to many other viral diseases such as rotavirus and coronavirus causing neonatal diarrhoea; bovine rhinotracheitis (IBR) and bovine diarrhoea (BVD) (*Borghese and Moioli, 2011*).

Rotavirus is an important aetiology causing neonatal diarrhoea in cattle and buffalo calves. In Asia, several cases were recognised more frequently in South Asia. In Sri Lanka, the virus was found to be the cause of 36.59% of diarrheic buffalo calves in the age from 1 to 60 days old. Interestingly, 12.5% of clinically healthy buffalo calves in the age from 1 to 150 days old also carried the virus (Sunil-Chandra and Mahalingam, 1994). In addition, the disease was also recognised in India, caused by both rotavirus group A and B with an average prevalence of 23.5% in buffalo calves (Nataraju *et al.*, 2009). In Pakistan, the prevalence was highest in winter (22.85%) and absent in the rest period (Khan *et al.*, 2009). In addition, coronavirus is another dangerous cause of diarrhoea in buffalo calves. In Pakistan, prevalence of the virus was highest in winter (17.14%) and absent in the rest period (Khan *et al.*, 2009). Even if these two viruses did not cause any health problem in adult buffaloes, the mortality in infected calves could be up to 20% or even higher if the patient does not receive appropriate care and treatment (*Khan et al.*, 2009).

Bovine viral diarrheic (BVD) is classified into type 1 and type 2, circulating in ruminants worldwide with clinical multiple manifestations including: diarrhoea, reproductive failure, congenital defect, respiratory disease, mucosal disease and haemorrhagic syndrome (Bhatia *et al.*, 2008). In Egypt, the disease was found in buffaloes, cattle, sheep, goats and camels. The highest prevalence of neutralizing antibody against BVDV was in buffaloes (52%), followed by cattle (49.2%) and lowest in sheep (27.5%). (Zaghawa, 1998). Furthermore, it was also identified in America *(Akhtar and Asif, 1996*).

Bovine herpesvirus 1 (BoHV-1) is a double-stranded enveloped DNA virus belonging to the family Alphaherpesvirinae, genus Varicellovirus. It is the causative agent of infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis (IBR/IPV), a worldwide disease that affects ruminants. BoHV-1 causes respiratory and reproductive symptoms, abortion, vulvovaginitis, encephalitis and fetal death. The virus is not always restricted to its natural host species. Animals such as goat, sheep, red deer and reindeer were successfully infected with BoHV-1 under experimental conditions (Thiry *et al.*, 2007). In addition, Mediterranean buffalo have been shown to be sensitive to BoHV-1 by experimental infection (Thiry et al., 2007), and by natural infection (Fusco *et al.*, 2015).

And what about influenza viruses ? We found only a single paper addressing the hypothesis of influenza viruses infections in buffaloes (Tajik et al., 2019). Its aim was to evaluate the prevalence of

H9N2-specific antibodies among water buffaloes. To this end, blood samples were obtained from 80 randomly selected water buffaloes, 40 cases of which were obtained in the winter months, and 40 cases were sampled in the spring months. The presence of H9N2-specific antibody was determined by hemagglutination inhibition method. The antibody was diagnosed in 14 animals. There were no significant differences between the two genders and between different age groups in terms of antibody prevalence. The presence of the antibody had a seasonal pattern; in this regard, all positive cases were found in the winter months. The question remaining is whether or not other teams looked at influenza viruses in buffalo medicine. In brief, it seems that infections of buffaloes by influenza viruses seem possible but their extend is still to be determined.

It is clear that diseases also have severe effects on buffalo production, and also threaten public health since some of those are high risk of zoonotic disease such as rotavirus group A (Chigor *et al.*, 2014). Unfortunately, in Southeast Asia, attention on these diseases has been overwhelmed by FMD for many years, leading to inappropriate awareness of the disease control and prevention at both national and regional levels. This circumstance provides good conditions for wide spreading of the trans-boundary diseases throughout the region. Specifically, the present animal disease control systems still manifest several limitations in both policy and the nature of disease diagnostic technology.

In order to obtain better results in control and prevention of trans-boundary diseases in domestic water buffalo in each individual country and international levels, it is key to establish regulation system, which satisfies requirements of both biosafety and market. Thus, besides an appropriate modification of the policy systems, it is crucial to develop new diagnostic tests, which can be used simply on the field to detect viral infections rapidly in domestic water buffaloes, so that infected animals, including asymptomatic individuals, can be identified and eliminated timely at the place of departure to prevent disease expansion.

2. Interferons-induced Mx proteins: the potential biomarker for screening early viral infection in vertebrates

In this section, we do not refer to recent reviews on the subject. This is because we preferred to assign all kinds of “Mx-knowledge” to their respective original contributors.

In 1962, Jean Lindemann and his colleagues accidentally discovered that the inbred strain of laboratory "A2G" mice resisted typically influenza A virus doses, which were lethal for the other mouse strains (Lindenmann, 1962). This resistance phenotype is called myxovirus resistance and the corresponding acronym, Mx and its variants were created including Mx (+/+), Mx (+/-) and Mx (-/-). The phenotyping of the inbred offspring in F1 and in F2 from the direct cross between susceptible and resistant mice strains demonstrated an autosomal and dominant mode of transmission of the Mx (+) phenotype (Lindenmann, 1964). In addition, the virus resistant strains among the offspring were rather

exceptional, it manifested only in the inbred strains A2G and SL/NiA (Abujiang *et al.*, 1996, Haller *et al.*, 1986, Lindenmann and Klein, 1966). Towards the end of the 1970s, it was found that the expression of the resistance phenotype depends on the concomitant presence of type I interferons (Haller *et al.*, 1979, Haller *et al.*, 1980). For example, a protein of 72 kDa was illustrated in the electrophoresis of protein lysates derived from either macrophages stimulated with type I interferons or infected embryonic cells, originated from resistant mice meanwhile the protein was absent in protein lysates from unstimulated macrophages or infected embryonic cells from susceptible mice (Horisberger *et al.*, 1983). Shortly thereafter, the mouse Mx protein, which was expressed under stimulation of type I interferons, was located in the nucleus of resistant mouse cells (Staeheli and Haller, 1985, Dreiding *et al.*, 1985). During the 1980s, the rapid development of molecular biology led to the first discovery of mouse genes encoding Mx proteins (Staeheli *et al.*, 1986c). The cloning techniques of cDNA were then implemented to express these proteins in other cell lines. In doing so, new experimental preparations have emerged that allow the testing of Mx protein functions including antiviral property under standardized conditions (Staeheli *et al.*, 1986b).

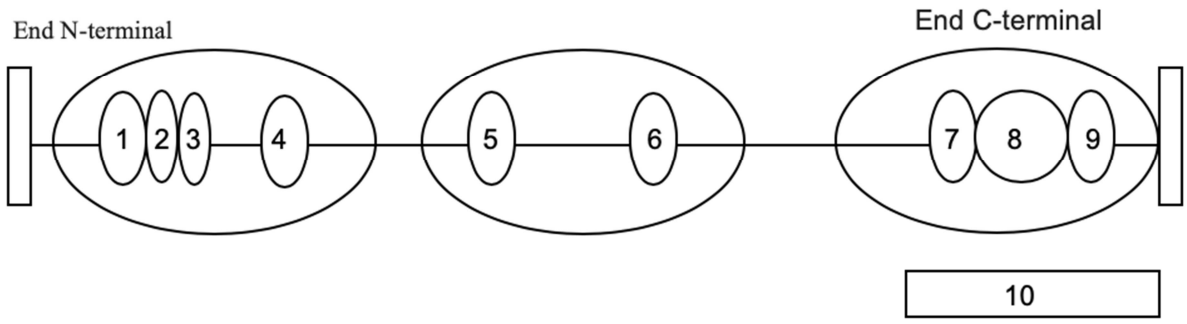

Figure 20: Schematic presentation of the structure of Mx proteins in general.

Notes: 1) GTP binding motif; 2) GTP binding pattern; 3) GTP Link Pattern; 4) Signature of dynamins; 5) Self-assembly domain; 6) Inter and intramolecular interaction domain 7) Leucine zipper pattern 1; 8) Nuclear Location Signal; 9) Leucine Zipper pattern 2; 10) GTPase Effector Domain (GED).

2.1. Structural characteristics of Mx proteins

By comparing the primary amino acid sequences of the different Mx proteins, we find that their structure has constants, in particular a tripartite GTP binding motif and a "dynamin" signature (Fig. 20). Proteins that aggregate GTP play an important role in many cellular processes. They participate in the biosynthesis of certain proteins, the transduction of cellular signals, vesicular transport, and the control of cell proliferation and differentiation (Bourne *et al.*, 1990). It may therefore, a priori, be assumed that the Mx proteins could exert one or the other of these functions. Taking into consideration

their functions and structural homologies, GTP-binding proteins can be classified into 5 superfamilies, among which the superfamily is known as "high molecular weight GTPases", whose dynamins are part (Van Der Bliek, 1999). Originally, dynamins have been described as being able to bind microtubules (Paschal *et al.*, 1987). Subsequently, a substrate function for protein kinase C (PKC) in neuronal tissue has been suspected (Robinson *et al.*, 1994), as well as participation in the process of endocytosis. The latter function has been demonstrated by the homology of sequences of the Drosophila shibire gene and genes encoding the dynamins (Van der Bliek and Meyerowrtz, 1991, Chen *et al.*, 1991).

A study of the behavior of the shibire fly reinforces the theory that the mammalian homologue of the shibire gene participates in the formation of clathrin-coated vesicles at the level of the plasma membrane (van der Bliek *et al.*, 1993, Damke *et al.*, 1994). At present, dynamins appear to be a conserved family of proteins involved in many cellular processes such as membrane remodeling, vesicular trafficking, and the division of organelles and cells (Praefcke and McMahon, 2004, Hinshaw, 2000, Song and Schmid, 2003).

The subfamily of dynamin-like proteins belongs to the family of dynamins. These dynamin-like proteins are characterized by: a) a high molecular weight (60-100 kDa), b) a tripartite GTP binding motif, c) an intermediate domain and d) a GTPase effector domain, formerly defined as the coiled-coil domain. In contrast to the "classic" members of the dynamin family, these proteins are free from: a) a pleckstrin-homology (PH) domain located between the GTPase domain and the intermediate domain and b) an arginine-rich domain traditionally located at the level of the carboxy-terminal end.

Considering the different functions, the so-called dynamin-like proteins can be classified into 4 groups (Van Der Bliek, 1999) including: a) the proteins involved in vesicular transport (for example VPS1p in yeast Saccharomyces cerevisiae and dyn-1 in Caenorhabditis. elegans nematode); b) proteins involved in mitochondrial division (e.g. Dnm1P and Mdm1 in S. cerevisiae or DRP-1 in C. elegans or Drp1 / DLPI-OPA1 in all vertebrates (Gammie *et al.*, 1995, Imoto *et al.*, 1998, Smirnova *et al.*, 1998, Alexander *et al.*, 2000, Delettre *et al.*, 2000), c) belonging to the group of plant dynamins (e.g. phragmoplastin / PDL, ADL1, ADL2a, ADL2b and ADL3 in brassicaceae A. thaliana (Gu and Verma, 1996, Gu and Verma, 1997, Kang *et al.*, 1998, Park *et al.*, 1997) and d) interferon-induced proteins (e.g. Mx proteins, p47 guanylate-binding proteins or p-67-guanylate-binding proteins (Staeheli and Haller, 1985, Boehm *et al.*, 1998).

2.1.1.Mx proteins are large GTPases.

The members of this superfamily are characterized by: (a) a high molecular weight, (b) a tripartite GTP-binding consensus sequence, and (c) a common GTP hydrolysis mechanism (Bourne et al., 1990, Dever *et al.*, 1987). The Mx proteins are characterized by (a) a molecular mass ranging from 70 to 80 kDa (Horisberger *et al.*, 1990b), (b) a GTP binding site consisting of a highly conserved tripartite sequence GXXXXGKST/T, DXXG and T/NKXD (X = any amino acid) located at the

amino-terminus (Pitossi *et al.*, 1993), (c) a relatively low affinity for GTP, and (d) a GTPase activity closely dependent on the substrate concentration. The murine protein Mx1 and the human protein MxA are able to hydrolyze the GTP in GDP; given the K_m value of the GTP hydrolysis reaction, it is likely that a high concentration of substrate is required to reach a maximum reaction rate. The dissociation constant, Kd, of MxA protein for the GDP is five times greater than for the GTP. This suggests that after hydrolysis of the GTP, the newly formed GDP is rapidly replaced by a new GTP molecule. Site-directed mutagenesis experiments evaluated the correlation between this enzymatic activity and antiviral activity. When a mutation removes the GTP binding consensus motif, the antiviral activity is abolished. This observation suggests that binding and / or hydrolysis of GTP is crucial for the antiviral activity of Mx proteins (Melén and Julkunen, 1994, Pitossi et al., 1993, Toyoda *et al.*, 1995). Subsequent in vitro experiments have shown that GTP binding is more important than its hydrolysis for the exercise of antiviral function (Schwemmle *et al.*, 1995b). Since the functioning of high molecular weight GTPases is regulated by a conformational change induced by GTP or GDP binding, it can be assumed that the same is true for Mx proteins.

2.1.2.Mx proteins are dynamins

The so-called "dynamin-like" proteins were discovered thanks to the sequence homology of their GTPase domain and their intermediate domain with homologous domain sequences in dynamins (Van Der Bliek, 1999). However, the dynamin-like proteins are free of (a) the pleckstrin domain and (b) the arginine-rich domain, which is usually present in typical dynamins. The presence of two small segments (residues 650-680 and residues 710-740) adopting a so-called coiled-coil conformation makes it possible to assume that the C-terminal domain of dynamins (GED for GTPase Effector Domain) is endowed with assembly faculties (Lupas *et al.*, 1991, Okamoto *et al.*, 1999). Moreover, the interaction between GED and the GTPase domain demonstrates that the coiled-coil segments are well involved in protein-protein interactions (Muhlberg *et al.*, 1997, Sever *et al.*, 1999, Smirnova *et al.*, 1999, Lupas et al., 1991). In fact, in dynamins, GED promotes oligomer self-assembly that exacerbates GTPase activity; GED can therefore be defined as an activating domain of GTPase (Muhlberg *et al.*, 1997, Sever *et al.*, 1999, Hinshaw, 2000).

Sequence analysis of the C-terminal end of several Mx proteins reveals the systematic presence of coiled coil motifs, called leucine zippers (Hirst *et al.*, 1996). Patterns of this nature are known to be responsible, inter alia, for the dimerization of many transcription factors and other regulatory proteins (Landschulz *et al.*, 1988). Fig. 21 and 22 show a strong conservation of this sequence between species, which suggests an important functional role (Brocchieri and Karlin, 2000). In 1992, Melen *et al.* demonstrated that leucine zipper motifs play a role in the oligomerization of Mx proteins (Melen *et al.*, 1992a). Since Mx proteins form aggregates both in vivo and in vitro, their purification is difficult (Dreiding et al., 1985, Ponten *et al.*, 1997, Weitz *et al.*, 1989). As a result, the question of the important function of oligomer formation remains unresolved

(Di Paolo *et al.*, 1999b). At present, some authors postulate that an important role is played by the self-assembling domain located at the amino-terminal end of the Mx proteins (Nakayama *et al.*, 1993). Meanwhile, some others point to the involvement of the carboxy-terminal end for the formation of homotypic bonds (Melén and Julkunen, 1997, Ponten et al., 1997, Di Paolo et al., 1999b, Melen *et al.*, 1992b, Schwemmle *et al.*, 1995a). In 1999, Di Paolo and colleagues proposed the following model to explain the implication of the leucine zipper motif in the oligomerization of the Mx protein: the folding back of the C-terminal region, with its leucine zipper motif (region 1), on its proximal domain of interaction (region 2) would favor the homotypic interaction of the Mx protein with a second Mx protein; this association is favored by the intermolecular interaction domain (region 3) of the second Mx protein. Since the intermolecular interaction domain (region 3) of the first Mx protein is not involved in the formation of dimers, it can interact with a third Mx protein and the formation of oligomers becomes possible (Melen *et al.*, 1992b, Schumacher and Staeheli, 1998) (Fig. 23) (Di Paolo *et al.*, 1999a), included for generating large aggregates of more than 30 proteins (Richter *et al.*, 1995). The examination of oligomeric structures by electron microscopy reveals a ring, stick or spiral shape (Nakayama et al., 1993, Kochs *et al.*, 2002a).

Position	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e	f	g
Murine MX1	Q	I	P	L	I	I	Q	Y	F	I	L	K	T	F	G	E	E	I	E	K	M	M	L	Q	L	L	Q	D
Murine MX2	H	V	P	L	I	I	Q	Y	F	I	L	K	M	F	A	E	R	L	Q	K	G	M	L	Q	L	L	Q	D
Rat MX1	Q	I	P	L	I	I	Q	Y	S	I	L	Q	T	F	G	Q	E	M	E	K	A	M	L	Q	L	L	Q	D
Rat MX1	H	I	P	L	I	I	Q	Y	F	I	L	K	M	F	A	E	K	L	Q	K	G	M	L	Q	L	L	Q	D
Rat MX1	H	I	P	L	I	I	Q	Y	F	I	L	K	M	F	A	E	K	L	Q	K	G	M	L	Q	L	L	Q	D
Duck MX	Q	I	P	L	I	I	L	S	A	V	L	H	D	F	G	D	N	L	Q	S	S	M	L	H	L	L	Q	E
Poultry MX	Q	I	P	L	I	I	L	S	T	V	L	H	D	F	G	N	Y	L	Q	S	S	M	L	H	L	L	Q	G
Human MXA	H	I	P	L	I	I	Q	F	F	M	L	Q	T	Y	G	Q	Q	L	Q	K	A	M	L	Q	L	L	Q	D
Human MXB	Q	I	P	F	I	I	Q	Y	F	M	L	R	E	N	G	D	S	L	Q	K	A	M	M	Q	I	L	Q	E
Porcine MX1	H	I	P	L	I	I	Q	F	F	I	L	R	T	F	G	Q	Q	L	Q	K	S	M	L	Q	L	L	Q	N
Bovine MX1	H	I	I	L	I	I	Q	F	F	V	L	R	T	Y	G	E	Q	L	K	K	S	M	L	Q	L	L	Q	D
Sheep MX	H	I	P	L	I	I	Q	F	F	V	L	R	T	Y	G	E	Q	L	K	K	S	M	L	Q	L	L	Q	D
Canine MX	H	I	P	L	I	I	Q	Y	F	I	L	Q	V	Y	G	Q	K	L	Q	N	G	M	L	Q	L	L	Q	D

Figure 21: Absolute retention of leucine zipper 1 in Mx proteins

Position	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e	f	g
Murine MX1	K	K	F	L	K	R	R	L	L	R	L	D	E	A	R	Q	K	L	A	K	F
Murine MX2	R	K	F	L	K	E	R	L	A	R	L	A	Q	A	R	R	R	L	A	K	F
Rat MX1	K	K	F	L	K	R	R	L	L	R	L	D	E	A	Q	R	K	L	A	K	F
Rat MX1	R	R	F	L	K	E	R	L	A	R	L	A	Q	A	Q	R	R	L	A	K	F
Rat MX1	R	R	F	L	K	E	R	L	A	R	L	A	Q	A	Q	R	R	L	A	K	F
Duck MX	R	N	Y	L	S	G	R	V	N	R	L	S	K	A	Y	Q	C	L	K	D	F
Poultry MX	Q	K	L	L	T	S	R	I	S	H	L	N	K	A	Y	Q	Y	L	V	D	F
Human MXA	R	K	F	L	K	E	R	L	A	R	L	T	Q	A	R	R	R	L	A	Q	F
Human MXB	R	R	I	F	K	E	R	I	Y	R	L	T	Q	A	R	H	A	L	C	Q	F
Porcine MX1	R	K	F	L	K	E	R	L	M	R	L	T	Q	A	R	R	R	L	A	K	F

Bovine MX1	R	K	F	L	K	E	R	L	E	R	L	T	R	A	R	Q	R	L	A	K	F
Sheep MX	R	K	F	L	K	E	R	L	E	R	L	S	R	A	R	Q	R	L	A	K	F
Canine MX	R	K	F	L	K	E	R	L	A	R	L	A	Q	A	R	R	R	L	A	K	F

Figure 22: Absolute retention of leucine zipper 2 in Mx proteins.

Notes : The table consists of an alignment of the amino acid sequences characteristic of the leucin zipper motifs of the carboxy-terminal end of the Mx proteins. The leucin zipper motifs 1 and 2 are represented with the positions of the amino acids important for the amphipatic helix.

The carboxy-terminal end of the Mx protein would also include a domain involved in the activation of GTPase activity (Flohr *et al.*, 1999, Muhlberg et al., 1997, Warnock and Schmid, 1996). Therefore, it appears that the GED of dynamin family members and the dynamin-like protein subfamily members act as a GTPase activating protein (GAP) (Sever *et al.*, 1999); thus, several series of hydrolysis of GTP could be undertaken without intervention of auxiliary factors. In this context, the proposed model postulates that the aforementioned backward folding is followed by an interaction of this accessory GTPase domain with the tripartite site serving to link the GTP that is in the N-terminal domain, which would form the active center of the enzyme (Schwemmle *et al.*, 1995a).

Mx proteins are classified within the family of dynamin-like proteins, since they do not possess, among others, PH domain (Hinshaw, 2000). This promotes the binding of phosphatidylinositol 4,5-bisphosphate in dynamins, which is crucial for membrane localization and for the intervention of dynamins in receptor-dependent endocytosis (Salim *et al.*, 1996). Thus, conventional dynamins are able to oligomerize and are able to bind lipid membranes (Sweitzer and Hinshaw, 1998, Takei *et al.*, 1995, Takei *et al.*, 1998). However, DLP1, which is a member of the dynamin-like proteins family and therefore free of the PH domain, preserves its binding capacity to lipid membranes (Yoon *et al.*, 2001). Thus, it is sought to know if the Mx proteins, despite the absence of the PH domain, also have a faculty of binding to lipid membranes. In 2002, it was proved in vitro that MxA protein, retains the ability to bind and tubulate artificial liposomes (Accola *et al.*, 2002).

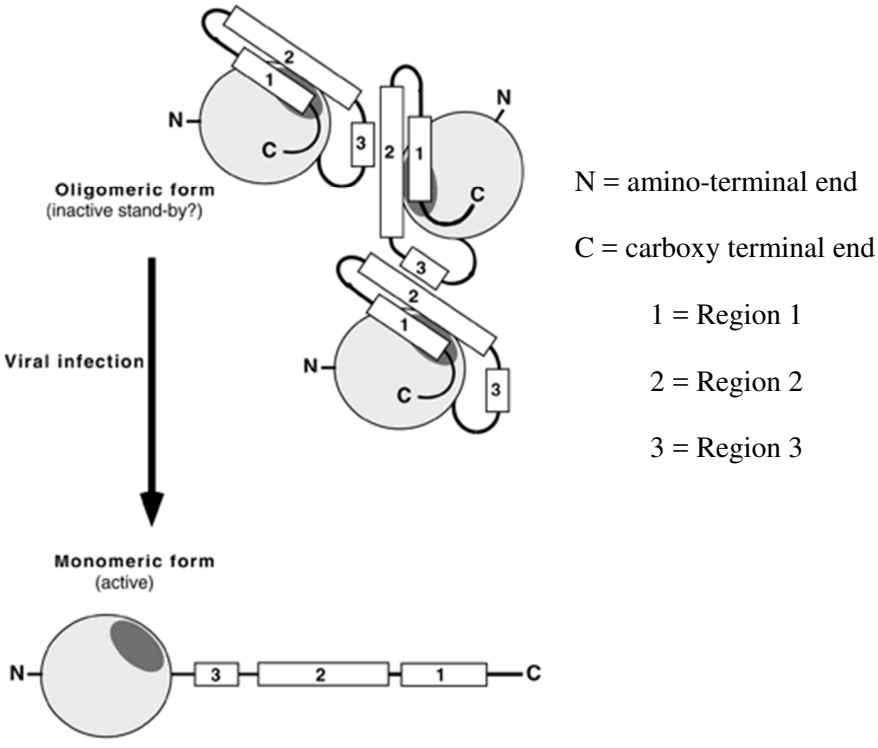

Figure 23: Mx-Mx interaction model involving the carboxy-terminal end.

2.2. The structural specificities of antiviral Mx proteins

Some allelic versions of Mx proteins are effectors of innate immunity that protect the body against viral aggressions (Lee and Vidal, 2002). The integrity of the amino-terminal end, the central interactive domain and the carboxy-terminal end are essential for the exercise of this antiviral function. For example, the antiviral function depends on the ability of the N-terminus to aggregate the GTP (Pitossi *et al.*, 1993, Melén and Julkunen, 1994, Schwemmle *et al.*, 1995a, Toyoda *et al.*, 1995) and the prevention of replication of the *Thogoto viru*s assumes the presence of the entire interactive core domain (Kochs and Haller, 1999b, Toyoda et al., 1995, Flohr et al., 1999). Most authors agree, however, that direct hardware support for antiviral function is GED (Kochs, 1998). Strangely, the C-terminal end is little conserved (Lee and Vidal, 2002), but it is the same, as compared the C-terminal sequences of the dynamines in general (Lee and Vidal, 2002). In the case of "non-Mx" dynamins, a direct link has already been established between specific GEDs, subcellular localization and specific functions (Lee and Vidal, 2002, Floyd and De Camilli, 1998). It is therefore possible that the GED of the Mx proteins evolved in parallel with one or more specific viruses of the species in question (Lee and Vidal, 2002).

Table 2: Inventory of known mutations that invalidate antiviral function.

Mx protein	Mutation	Mx Variant	Subcellular localization	Anti-viral activity	References
1) Human MxA	Position 645- second leucine zipper pattern - exchange of a glutamic acid by an arginine	MxA E645R	Cytoplasm	Maintenance of anti-influenza activity associated with loss of anti-VSV activity	(Zurcher *et al.*, 1992).
2) Murine Mx1	Position 614- nuclear localization signal- exchange of a glutamic acid by an arginine	Mx1 E614R	Cytoplasm	Loss of anti-influenza activity	(Noteborn *et al.*, 1987, Zurcher *et al.*).
	Position 619-second leucine zipper motif perturbation of the α-helix - exchange of a leucine by a proline	Mx1 L691P	nuclear	Loss of anti-viral activity	(Lee and Vidal, 2002).
3) Poultry Mx1	Position 631-exchange of a serine with an arginine	Mx1 S631N	Cytoplasm	Loss of anti-VSV activity.	(Ko *et al.*, 2004)
4) Rat Mx2	Position 588 - exchange of an arginine with a cysteine; at position 630 exchange of a lysine with a histidine	Mx2 R588C MX2 H630K	Cytoplasm	Loss of anti-VSV activity.	(Johannes *et al.*, 1997)
5) Rat Mx3	Position 588- exchange of a cysteine by an arginine; at position 630- exchange of a lysine with a histidine	Mx3 C588R Mx3 K630H	Cytoplasm	Partial restoration of anti-VSV activity	(Johannes *et al.*, 1997)
6) Porcine MX1	Nucleotide position 2064- deletion of 11 bp inducing elongation of 23 aa	Mx1	Cytoplasm	Partial loss of anti-influenza activity	(Morozumi *et al.*, 2001, Palm *et al.*, 2007)

Table 2 lists the different GED directed mutagenesis experiments that have been performed to date and their effect on antiviral activity. It is indicated that most alterations in genetic material can cause a complete loss of antiviral activity including point mutations, suggesting that the exercise of a given antiviral function is subject to specific structural constraints (Garber *et al.*, 1993, Melén and Julkunen, 1994).

2.3. Genomic organization & control of expression at Mx loci

The murine Mx1 transcript and the corresponding murine Mx1 protein were described first. Rapidly, it appeared that the pool of Mx transcripts of the mouse consisted of the transcription products of two distinct genes, denoted Mx1 and Mx2. Since then, homologous genomic sequences have been described in all tested species (Horisberger and Gunst, Müller and Brem, 1991), for example a fish species (Staeheli *et al.*, 1989), *Rattus norvegicus* (Meier *et al.*), *Homo sapiens* (Staeheli and Haller, 1985, Aebi *et al.*, 1989), *Bos taurus* (Horisberger, 1988), *Ovis aries* (Charleston and Stewart, 1993), *Equus caballus* (Chesters *et al.*, 1997, Hicks *et al.*, 2003), *Canis familiaris* (Nakamura

et al., 2005), *Felis catus* (Horisberger *et al.*) and *Sus scrofa* (MÜLLER *et al.*, 1992). Table 2 lists the access codes to the relevant genomic sequences. The complete genomic organization of murine Mx1 and Mx2 genes (Hug *et al.*, 1988, Asano *et al.*, 2003), human MxA (Ronni *et al.*, 1998), porcine Mx1 (Thomas *et al.*, 2006) and bovine Mx1 (Gérardin *et al.*, 2004) were published. The expression of the mx genesis is subordinated to the presence of type 1 interferons (Horisberger et al., 1983, Haller et al., 1979, Staeheli *et al.*, 1984, Horisberger, 1988, Staeheli *et al.*, 1986a), as confirmed by the presence of one or more GAAAN1-2GAA type motifs (C/G) in the corresponding promoters, so-called "interferon stimulated response element" reasons because of their sensitivity to interferons α/β (Hug et al., 1988, Horisberger *et al.*, 1990a, Gérardin et al., 2004, Schumacher *et al.*, 1994, Ronni et al., 1998, Collet and Secombes, 2001, Chang *et al.*, 1991b). Some promoters are provided with two ISREs (murine Mx1, porcine Mx1, human MxA) while others have only one (bovine Mx1, chicken Mx1, rainbow trout Mx1) (Thomas *et al.*, 2006). In addition, some promoters include "ISRE-like" motifs composed of two GAAA tetramers separated by more than two nucleotides (Ronni *et al.*, 1998, Gérardin *et al.*, 2004, Hug *et al.*, 1988). The exact functions of these sequences are not formally established, but assume that they are responsible for the fine modulation of the corresponding Mx gene transcription rate (Ronni *et al.*, 1998). Such motifs are present within the murine Mx2 gene promoter, human MxA, bovine Mx1 and porcine Mx1. In some cases, the Mx promoters show one or the other singularity; for example the absence of TATA and CCAAT boxes in humans, pigs or cattle (Gérardin *et al.*, 2004, Ronni *et al.*, 1998, Thomas *et al.*, 2006, Chang *et al.*, 1991b) or the presence of the TATA motif without CCAAT motif in rainbow trout and in mice (Collet and Secombes, 2001, Hug *et al.*, 1988). Finally, sometimes more proximal regulatory motifs are found, such as the first intron of the human MxA gene that significantly influences the level of expression of the corresponding protein (Ronni *et al.*, 1998), or an ISRE-like element in the first intron of the bovine Mx1 gene that could also control the level of expression of the bovine Mx1 protein (Gérardin *et al.*, 2004). On a kinetic level, the accumulation of Mx mRNAs is rapid and transient. A peak is reached as early as the eighth hour after exposure to type I interferons and the basal level is restored 24 to 48 hours after stimulation. At peak, Mx mRNAs can represent up to 0.1% of the total polyadenylated RNA. The corresponding Mx protein concentration reaches a maximum 24-48 hours after stimulation but it's very low basal level is not restored until several days later due to a relatively long half-life (> 48 hours) (Staeheli *et al.*, 1986a). This particular expression pattern explains why Mx proteins are good biomarkers for the presence of type 1 interferons (Fäh *et al.*, 1995, Jakschies *et al.*, 1990). It should be noted that interferon gamma and other cytokines, on the other hand, do not seem to significantly influence the level of expression of mx genes (Aebi et al., 1989, Bazzigher *et al.*, 1992, Staeheli et al., 1984, Staeheli et al., 1986a).

2.3.1. Mouse Mx locus

Historically, the Mx system has been found in mice following fortuitous detection of the innate resistance of the inbred strain of A2G mice at typically influenza A virus doses, which was lethal for

other laboratory mice (Lindenmann, 1962). This feature of resistance to Orthomyxovirus has been called Mx + (Lindenmann, 1964). Later, a correlation could be established between this innate resistance trait and the type 1 interferon-inducible expression of a ~ 72.5 kDa protein (Staeheli *et al.*, 1983).

The Mx1 gene

The two mouse *mx* genes are located on the distal arm of chromosome 16 (Staeheli *et al.*, 1986d, Reeves *et al.*, 1988). The Mx1 gene consists of 14 exons spread over a 55,000 base pair chromosomal segment, which is expected to encode a protein of 631 amino acids (Hug *et al.*, 1988). The first intron is 30,000 bases long and separates the 29-base pair long non-coding exon 1 (beginning of the UTR5 ') from the exon 2 containing successively (from 5' to 3') the continuation and the end of the non-coding sequence (end of the UTR5'), the start codon and the start of the coding sequence. The latter is interrupted by 12 introns. The length of the different exons varies between 77 and 258 bases. The last exon, which encodes the 76 carboxy-terminal amino acids, is composed of 1339 bases and includes the entire 3 'untranslated region (UTR3'). Northern blots made from transcripts extracted from Mx (+) cells and Mx1 (+) (-) specific probes reveal a band of 3.5 kb, which corresponds to the expected transcript (Hug *et al.*, 1988). The promoter region of the Mx1 gene of the mouse comprises 5 GAAANN type sequences (or its reverse complement).

Motifs of this type are present in all genes whose transcription is stimulated by interferons or by viruses. In the mouse Mx1 promoter, as in all promoters inducible by the presence of type I interferons, these elements are arranged in tandem, resulting in a purine-GAAAN (N/-) GAAA (C/G) -pyrimidine (or its inverse complement) called interferon sensitive response element (ISRE) (Hug *et al.*, 1988). As the mouse Mx1 protein is expressed, it is found exclusively in the nucleus where its topographic distribution generates a visual "in points" or even finely granular (Dreiding *et al.*, 1985, Horisberger *et al.*, 1983). The fine "Mx" granules are juxtaposed with dynamic subnuclear structures whose composition, number and size varies throughout the cell cycle and depends on various stimuli, PLM-NB (promyelocytic leukaemia protein-nuclear bodies (Chelbi-Alix *et al.*, 1995, Trost *et al.*, 2000, Engelhardt *et al.*, 2001). Interestingly, some components of PLM-NB are modified by SUMOylation (Hodges *et al.*, 1998, Matera, 1999, Maul *et al.*, 2000, Sternsdorf *et al.*, 1997) and one publication suggests that mouse Mx1 interacts with SUMOylation machinery (Engelhardt *et al.*, 2001).

The Mx2 gene

In mice always, Northern blots systematically show a second transcript inducible by type I interferons. In fact, a specific cDNA probe Mx1 also hybridizes to a mRNA of 2.5 kb. The transcript thus revealed comes from the Mx2 gene. Gels from Northern blotting always show a thick band around 2.5 kb, which is consistent with the presence of two polyadenylation signals. Aligning the

cDNAs Mx1 and Mx2 reveals a sequence identity of 73%. Oddly enough, as in the case of the Mx1 gene, most of the inbred laboratory strains carry a point mutation in the Mx2 gene that shifts its reading frame and generates an inactive Mx2 protein. In wild-type mice, on the other hand, the allele that leads to a functional Mx2 protein is common (Hug *et al.*, 1988). Like the Mx1 gene, the Mx2 gene is composed of 14 exons supposed to encode a protein of 655 amino acids (Jin *et al.*, 1999). With the exception of the 5 'and 3' ends of the cDNA, the similarity of the two genes is striking, it probably reflects ancestral duplication. The promoter region of the Mx2 gene is located between nucleotides -578 and +1. The analysis of the region between nucleotides -97 and -53 shows an ISRE-like proximal sequence. This specific motif makes it possible to predict that the induction of the gene expression is subordinated to the presence of type I interferons (Asano *et al.*, 2003) and, in fact, the temporal pattern expression of Mx1 and Mx2 protein are relatively the same each other. However, the Mx2 protein of the mouse is expressed in 3T3 cells, it is found exclusively in the cytoplasm (Jin *et al.*, 1999).

2.3.2. Human Mx locus

The exposure of human cells to type I interferons induces the expression of several new proteins. Immunoprecipitation of these with mouse anti-Mx1 antibodies reveals the presence of homologous human proteins. The use of mouse cDNA Mx1 as a hybridization probe makes it possible to put two types of human cDNAs Mx in evidence. Sequence analysis shows that the corresponding mRNAs are derived from the transcription of two different human mx genesthat have been named MxA and MxB. The sequence similarity between murine human Mx proteins is between 56 and 77%. The comparison of the sequences also reveals a very great similarity between the murine Mx2 protein and the human MxA protein (Aebi *et al.*, 1989). Western blotting analysis reveals the presence of two MxB proteins (Kozak, 1987). The context nt-3/nt + 4 (CACATGT) of the first start codon is not ideal for the initiation of translation, which explains that it also starts from the second start codon, hence the two proteins identifiable after gel migration. MxA protein is identifiable exclusively in the nucleus. The pattern of expression is more complex for the MxB. It is often located exclusively in the cytoplasm, but in some cells (macrophages, for example), it is observed in both the cytoplasm and the nucleus. A nuclear localization signal has also been identified in the N-terminus (Melén *et al.*, 1996). MxA and MxB genes are located on the long arm of human chromosome 21 (Horisberger *et al.,* Raziuddin *et al.*, 1984).

Promotor

The expected ISRE-like consensus elements are present at the promoter level (Chang *et al.*, 1991b). The promoter analysis shows: a) the existence of three ISRE elements (during the transfection experiments, at least one activity is demonstrated for both proximal elements (Chang *et al.*, 1991a, Horisberger et al., 1990b); b) three possible elements of response to IL-6 (CTGGA), c) a potential binding site for NFκB, and d) a potential Sp1 factor binding site (Ronni *et al.*, 1998). The proximal

ISRE element appears to be essential for the induction of transcription by α interferons, while the second element probably stimulates the general inducibility of the promoter (Ronni *et al.*, 1998). The promoter region shows a polymorphic site (nucleotide -88) located within a consensus sequence resembling ISRE elements. This SNP affects the expression of MxA (Nakade *et al.*, 1997, Hijikata *et al.*, 2001). The first intron presumably has an additional transcription enhancer that can influence the level of expression (Ronni *et al.*, 1998).

Interaction

Protein interaction studies describe a MxA protein capable of binding cytoskeletal structures (Horisberger, 1992). However, the use of cytoskeletal disrupting agents does not alter the intracellular distribution of MxA protein; this makes it possible to hypothesize a transient interaction between the protein and the elements of the cytoskeleton in vivo (Horisberger, 1992). Immunofluorescence and subcellular fractionation techniques demonstrate an association of MxA protein with the smooth endoplasmic reticulum. Surprisingly, electron microscopy demonstrates that the expression of a mutant MxA goes hand in hand with a substantial proliferation of the smooth endoplasmic reticulum. Thus, it appears that the human protein is involved in the remodelling of this membrane compartment (Accola et al., 2002, Kochs *et al.*, 2005). Immunofluorescence analysis also reveals colocalization of MxA protein with markers of endosomes, lysosomes and cis-Golgi; this is a strong indicator for additional association with these organelles (Stertz *et al.*, 2006). The Western Blot analysis of the different samples obtained by differential centrifugation demonstrates that approximately 30% of MxA proteins are found in the microsomal fraction and 70% in the post-microsomal supernatant (Stertz *et al.*, 2006).

2.3.1. Cattle Mx locus

The primordial observation of Mx-like proteins in the bovine species was carried out in MDBK cells (Madin-Darby bovine kidney) where stimulation by interferons α or β induces the cytoplasmic expression of two proteins recognized by an antiserum anti - MxA that are absent in untreated control cells (Horisberger, 1988, Horisberger and Gunst). Later, two cDNAs encoding Mx1 and Mx1a protein were isolated from a genomic library, corresponding to two splicing variants. The two reading frames encode a protein of 654 amino acids (Mx1) and another of 648 amino acids (Mx1a), the predicted molecular masses of which are 75.5 (Mx1) and 74.7 kDa for (Mx1a). The amino acid sequence similarity between ovine, human, murine and bovine Mx1 proteins is 93%, 73% and 63%, respectively.

The Mx1 bovine locus is localized on bovine chromosome 1 (Ellinwood *et al.*, 1999, Ellinwood *et al.*). Analysis of the bovine Mx1 gene reveals an organization in 15 exons spread over 60 kb and interrupted by 14 introns. The coding region extends from nucleotides 8-10 (ATG) located within the third exon to nucleotides 2290-2310 (TGA) located at the fifteenth exon. The 77

amino acids of the carboxy terminus and the entire UTR-3 'are encoded by the last 630 bp long exon (Gerardin *et al.*, 2004).

The promoter of the bovine Mx1 gene displays several putative binding sites for cytokines and other transcription factors. Thus, within this sequence (340 nucleotides upstream from the transcription start site), we describe: a) an ISRE motif, b) an ISRE-like element, c) a binding site for the NFκB, d) two binding sites for IL-6, e) two binding sites for SP1 and f) five GC-rich dishes. Like other interferon inducible promoters, this also shows GAAA stretches (Chang et al., 1991b, Hug et al., 1988, Reid *et al.*, 1989). On the other hand, the bovine Mx1 promoter is characterized by the absence of a TATA box and by the absence of CCAAT box (Gerardin *et al.*, 2004). As expected, the bovine Mx1 promoter can be induced by type I interferons. Like the ISRE-like element of the human MxA promoter, it is possible that the ISRE-like element of the bovine Mx1 promoter does not intervene in the control of the expression of the bovine Mx1 protein. However, computer analyses revealed the existence of a second ISRE-like motif located within the first intron; it could therefore play the role of a transcription stimulator and thus influence the level of expression of the bovine Mx1 protein. The nucleotide sequence and amino acid sequence of the second bovine gene/protein have been reported, however their characteristics remain unstudied (Babiker *et al.*, 2007).

2.3.2. Porcine Mx locus

The porcine Mx1 locus is located on chromosome 13 and encodes a protein of 663 amino acids (MÜLLER et al., 1992). The use of probes specific for murine cDNA Mx1 made it possible to identify two homologous transcripts in pigs (Staeheli *et al.*, 1986b). Northern blot analysis of the total RNAs obtained from cells treated with type I interferons reveals the presence of a 2.5 kb RNA whereas no hybridization signal is demonstrated in the preparations obtained from untreated control cells (MÜLLER et al., 1992). Similarly, circulating lymphocytes exposed to type I interferons and immunostained with a murine anti-mouse Mx polyclonal serum contain two proteins with apparent molecular masses of 73 and 76 kDa. Next, two porcine Mx cDNAs of different nature have been described; they differ by their restriction pattern and have been baptized Mx1 and Mx2. The differences between the porcine cDNAs Mx1 and Mx2 are such that it has been suggested from the start that they come from the transcription of two distinct genes (MÜLLER et al., 1992).

The porcine Mx1 gene consists of 15 exons and extends over about 25 kb; the coding sequence is composed of 13 exons. The Mx1 cDNA is 2537 bp long followed by a poly- (A) tail. The 77 amino acids of the carboxy-terminal end as well as the entire UTR-3 'are encoded by the last exon, which extends over 677 bp (Thomas *et al.*, 2006). The reading frame is spread between the start codon (position 101) and the stop codon (position 2090). The UTR-3 'consists of 446 bp, shows a polyadenylation signal and AUUUA pattern likely plays a role in the stability of the mRNA (Brawerman, 1989). The UTR-5 ', as for it, comprises the set of the first and the second exon as well

as a part of the third exon (Thomas *et al.*, 2006). The amino acid sequence deduced from the Mx1 cDNA consists of a 663 amino acid sequence which corresponds to a protein with a calculated molecular mass of 75.6 kDa (MÜLLER *et al.*, 1992).

The Mx2 cDNA consists of 2680 bp, including a long 2136 bp ORF (AB258432) from which it can be deduced that it encodes a protein of 711 amino acids. The porcine Mx2 gene consists of 14 exons, the start and stop codons being respectively present on exons 2 and 14. Porcine Mx2 has 69% and 68% identity with the human MxB and Mx2 bovine proteins, respectively. On the other hand, it can easily be discriminated against porcine Mx1 protein with which it shares only 55% identity (Morozumi *et al.*, 2009).

The porcine Mx1 promoter is strongly stimulated by type I interferons, this characteristic being associated with very low or even no transcription activity in the absence of type I interferons (Thomas *et al.*, 2006). This promoter is characterized by the presence of: a) two ISRE motifs in position -34 and -80 (the second, more proximal, motif could intervene in the activation of the gene (Thomas *et al.*, 2006, Ronni *et al.*, 1998), b) an ISRE- distal, which is equivalent in the promoter of the human MxA gene and in the promoter of the murine Mx2 gene (Ronni *et al.*, 1998, Asano *et al.*, 2003) (since this motif has no functional role in humans and in mice, it is also proposed to absence of intervention on the expression of the porcine Mx1 protein), c) the absence of a TATA motif, d) the absence of a CCAAT motif, e) the presence of putative binding sites for the NFKB, SP1 and IL-6, f) the presence of 3 GC-rich boxes and g) the presence of twelve sections of GAAA type (Thomas *et al.*, 2006).

2.4.Allelic variants at Mx loci

In principle, and like other genes, mx genes must have allelic variations. Given that some versions of these genes confer resistance to viral diseases, the inventory of allelic polymorphism Mx loci of livestock is likely to allow the identification of a more favourable allele in terms of resistance to viral diseases and therefore, to allow an assisted selection of the best breeders. No systematic inventory of individual variations has yet been undertaken on a large scale. Nevertheless, critical variations have already been identified.

2.4.1. Allelic diversity at the murine Mx locus

In mice, resistance to influenza virus infections is controlled by alleles present at the Mx locus, which is located on chromosome 16 (Haller, 1981, Staeheli and Haller, 1987). The study of the polymorphism of this locus in 5 laboratory mouse strains revealed the presence of at least 3 RFLPs (restriction fragment length polymorphisms). In mice, these genetic variations can be correlated with variations in resistance to influenza virus infections (Staeheli *et al.*, 1988, Staeheli et al., 1986b). The type I variant is demonstrated in mouse strains capable of producing a functional Mx1 protein. This allele consists of 14 exons and produces, in response to stimulation by α and β interferons, a 3.3 kb mRNA encoding a protein of 72 kDa (Staeheli *et al.*, 1986a, Staeheli *et al.*, 1986b).

The other two types of RFLPs have been identified in mouse strains susceptible to influenza virus infection and are unable to synthesize a functional Mx1 protein (Dreiding *et al.*, 1985, Horisberger *et al.*, 1983, Staeheli *et al.*, 1984). The type II variant produces small amount of a mRNA homologous to Mx mRNAs after activation by type I interferons (Staeheli *et al.*, 1986b). Within the coding region, there is a block of 424 nucleotides missing (absence of exons 9-11), which gives rise to a shift of the reading frame (Staeheli *et al.*, 1988). In addition, the coding sequence upstream of the deletion differs from that of the type I allele by the change of 6 nucleotides of which 5 remain silent, the fifth giving rise to the substitution Met-584-Thr (Staeheli *et al.*, 1988). This type II variant is the most common among inbred strains of laboratory mice. Mice possessing the type III variant are also unable to produce a functional Mx1 protein and are therefore susceptible to influenza virus infections (Haller *et al.*, 1987). After stimulation with type I interferons, the cells of these mice produce an apparently homologous mRNA of the classical Mx1 mRNA, displaying the expected size (Staeheli *et al.*, 1988). However, the sequence analysis reveals three major differences between the type I variant and the type III variant: a) a point mutation that changes the AAA codon (lysine at position 389) to TAA (stop codon); b) a point mutation that converts 12-position glutamic acid to lysine; and c) the inclusion of an additional 72 nucleotide exon in the 5 'non-coding region. In addition, 12 nucleotide substitutions are described whose 6 located in the coding region are silent and 6 others are located within the 3 'non-coding end (Staeheli *et al.*, 1988). Thus, the type III Mx1 allelic variant has a mutation that prevents the synthesis of a complete Mx1 protein of 72 kDa. Analysis of the cDNA sequence predicts a missing of truncated form, where nearly 40% of the carboxy terminal region (Staeheli *et al.*, 1988). Screening of many strains of laboratory mice shows that resistance to influenza virus infections is present in only a few inbred strains of laboratory mice, those derived from wild strains A2G and SL/NiA (Abujiang *et al.*, 1996, Haller *et al.*, 1986, Lindenmann and Klein, 1966), and the strains Cast/Ei more, recently. Functional allelic versions are therefore essentially found among strains of wild-type mice. Although these are not natural hosts of influenza A viruses, the Mx1 protein must play a role. Thus, it is likely that the Mx1 response targets a rodent-specific pathogen other than influenza A or influenza B.

The murine Mx2 sequence is very close to the Mx1 sequence; both genes are located in the same place on chromosome 16 (Staeheli and Sutcliffe, 1988). Most laboratory mice do not express Mx2 transcripts. A few inbred strains of laboratory mice synthesize Mx2 mRNA, but the corresponding ORF is interrupted by a supernumerary cytosine residue at position 1366, which imposes a frame shift. Thus, the Mx2 gene of the vast majority of inbred laboratory mouse strains is also non-functional (Jin *et al.*, 1999). On the other hand, the coding region of the Mx2 mRNA of wild-type NJL (Mus musculus musculus) and SPR (*Mus spretus*) strains differs from that of laboratory mouse strains by the absence of an alanine at position 1367, which suppresses a premature stop codon and gives rise to a transcript capable of encoding a Mx2 protein of 656 amino

acids (Jin *et al.*, 1999). Immunolabeling makes it possible to demonstrate a cytoplasmic location in the form of points for this Mx2 protein (Jin *et al.*, 1999). On the other hand, it has antiviral activity against VSV and against hantavirus but is inactive against infections with influenza viruses (Zürcher *et al.*, 1992, Jin *et al.*, 2001).

2.4.2. Polymorphism at the bovine Mx locus

In 1988, Ellinwood *et al* cloned the Mx cDNA of a crossbred Angus-Gelbvieh (Genbank accession Nos. AFO47692 and U88329). In doing so, they demonstrated an Mx1 variant with a deletion of 18 bp at position 171, which they suggested was a splice variant (ELLINWOOD *et al.*, 1998, Nakatsu *et al.*, 2004). Thanks to the development of a system allowing inducible expression of the Mx1 bovine protein, Baise *et al.* describe in 2004 a protection against VSV (Baise *et al.*, 2004). Based on nucleotide substitutions, Nakatsu *et al.* (2004) classified bovine Mx1 cDNA variants into two genotypes: (a) genotype I with a deletion within UTR-3' and (b) genotype II with different insertions. In order to determine the antiviral activity of the different variants, 3T3 cells are permanently transfected with either genotype and then infected with VSV. Cells expressing the bovine Mx1 protein have been shown to be significantly less often infected than cells do not expressing the bovine Mx1 protein. It can be deduced that the splice variant also exerts antiviral activity.

The bovine Mx2 cDNA (2381 bp) encodes a protein of 710 amino acids, the sequence data is available at accession number AF355147. By comparing the Mx2 cDNA in 11 bovine breeds, 17 nucleotide substitutions were identified, which gene 8 allelic variants (Babiker *et al.*, 2007). Sixteen substitutions are localized in the coding sequence, fourteens are silent and two others give rise to an amino acid change (Gly-302-Ser and Ile-354-Val) (Babiker *et al.*, 2007). Comparing the water buffalo cDNA Mx2 (2400 bp, coding region [285-2427], 710 amino acids, GenBank accession number EF052266) with the bovine Mx2 cDNA, 46 nucleotide substitutions were detected. Thirsty substitutions are silent and twelve induce a change of amino acid. In addition, there is an additional 9bp insertion at the 5'-UTR (Babiker *et al.*, 2007). Functional studies were carried out in 3T3 cells to compare the anti-VSV activity of several variants, which led to the demonstration of the superiority of one of the bovine allelic variants (Babiker *et al.*, 2007).

2.4.3. Polymorphism at the poultry Mx locus

The complete hen cDNA Mx1 sequence was determined in the *White Leghorn* breed (Bernasconi *et al.*, 1995). Later, by analysing this sequence in 15 different hen breeds, 25 nucleotide substitutions were detected, of which 14 are responsible for a change in amino acids. Thus, the cDNA Mx1 of the hen can be described as very polymorphic. In order to describe the anti-influenza activity of these different alleles, 3T3 cells were stably transfected with these different chicken Mx1 cDNAs. In this way, anti-influenza and anti-VSV activity have been associated with certain alleles (Benfield *et al.*, 2008). A first attempt of explanation advanced the change in amino acid at position 631 (Ser to

Asp) as determining for a positive or negative anti-viral response (Ko *et al.*, 2002). However, a second study examining this substitution was unable to replicate the same responses and strongly discourages selection to increase the frequency of the Asp631 allele of poultry populations (Ko *et al.*, 2002) as suggested by the first team (Li *et al.*, 2006).

2.4.4. Polymorphism at the porcine Mx locus

The complete porcine Mx1 cDNA was isolated and sequenced for the first time in 1992 by Müller *et al.*. The involvement of the carboxy-terminal end in the anti-viral function of the Mx protein was then described several times. In order to identify potentially (dys) functional Porcine Mx1 gene variants, the 14th exon was sequenced in different porcine breeds. Thus, two major alleles have been identified: (a) poMx1α, corresponding to the allele described by Müller *et al.* in 1992 and (b) poMx1β, corresponding to an allele characterized by a deletion of 11 bp (2063-2074) inducing a change of the reading frame and giving rise to a porcine Mx1 protein with an elongated carboxy-terminal end of 23 amino acids (MÜLLER et al., 1992). Additional studies highlight the existence of additional alleles; however, the anti-viral effect was only tested for two of them (Morozumi et al., 2001, ASANO *et al.*, 2002). Thus, Asano *et al.* (2002) described similar anti-VSV activity for both poMx1α and poMx1β alleles. However, in 2007, Palm *et al.* associated greater anti-influenza A activity with the poMx1α allele (Palm *et al.*, 2007).

2.5. The functions of Mx proteins

Within the superfamily of high molecular weight GTPases, Mx proteins are classified in the subfamily of dynamin-like proteins, which play several functional roles in the host.

2.5.1. MxA protein participates in membrane trafficking and remodelling

* Retention of endocytic material in cells expressing MxA protein

Dynamins perform several important functions, among which we can mention their intervention on membrane trafficking as well as on various other cellular transport processes. Dynamins are described as being able to form helices around the neck of nascent vesicles thus contributing to their scission of the parental membrane (Hinshaw and Schmid, 1995). Thus, dynamins are involved in receptor-dependent endocytosis. Mx proteins are part of the subfamily of dynamin-like proteins. Therefore, it is not surprising that (Jatiani and Mittal, 2004) described in 2004 an intervention of MxA protein in trafficking processes occurring during endocytosis. The retention of the endocytic material was observed in cells expressing MxA protein. This is consistent with previous observations describing the retention of viral cargo in infected cells expressing MxA protein (Kochs and Haller, 1999b). In addition to these studies, others describe changes in the distribution of endosomal/lysosomal membrane markers such as the mannose-6-phosphate receptor or Lamp-1 (Stertz *et al.*, 2006). Furthermore, the results of coimmunoprecipitation or pull-down tests indicate an

interaction of MxA proteins with dynamins. This interaction most likely interferes with the cellular transport processes usually regulated by dynamins (Jatiani and Mittal, 2004).

* MxA protein adheres to liposomes and tubulates them in vitro

A faculty common to all dynamins is their predisposition to self-assemble to form ring-like structures; this ability seems to be essential for their intervention during membrane remodelling (Hinshaw and Schmid, 1995, Carr and Hinshaw, 1997, McNiven, 1998, Hinshaw, 1999). Another important feature of dynamins is their ability to bind and tubulate lipids, such as at the time of scission of nascent vesicles (Sweitzer and Hinshaw, 1998, Takei et al., 1998). MxA proteins, like other members of the dynamin family, self-assemble to form oligomeric structures of high molecular weight resembling root-like structures or ring-shaped structures (Di Paolo et al., 1999b, Accola et al., 2002); this oligomeric form could induce the reorganization of the associated membranes (Stertz *et al.*, 2006). Nevertheless, the association between oligomerization and antiviral function has not yet been elucidated; especially, since MxA protein has been reported to be unable to self-assemble, which preserves its ability to inhibit the replication of *Thogoto virus* (Di Paolo et al., 1999b, Janzen *et al.*, 2000).

Co-sedimentation assays show that MxA protein is able to associate with lipids in vitro. In addition, when MxA protein is associated with spherical phosphatidylserine liposomes, long and often branched tubes are formed (Accola *et al.*, 2002). By analogy, some authors have come to assume that MxA protein interacts with subcellular membrane compartments in vivo, some of which are obviously places of viral replication (Reichelt *et al.*, 2004, Stertz et al., 2006). Consistent with this hypothesis, colocalization of MxA protein with markers of smooth endoplasmic reticulum, AMF-R and Syntaxin 17 is reported (Steegmaier *et al.*, 2000, Accola et al., 2002, Stertz et al., 2006). In electron microscopy studies, antibodies were used to recognize β'-COP-I, which is a subunit of the COP-I coat, located in the intermediate compartment between the Golgi apparatus and the endoplasmic reticulum, illustrated an evidence of an association between this intermediate compartment and MxA proteins (Stertz *et al.*, 2006). Subsequent studies show that the expression of a mutant MxA causes an expansion of the endoplasmic reticulum, hence the hypothesis that MxA protein is a member of the membrane traffic to or from the endoplasmic reticulum, which would explain why a mutant form alters normal membrane flux and causes an excessive expansion of the smooth endoplasmic reticulum (Accola *et al.*, 2002). The ability of fragmentation of lipid membranes of dynamins depends on the hydrolysis of GTP (Sweitzer and Hinshaw, 1998). In contrast, it has been demonstrated, in vitro, that the hydrolysis of GTP associated with MxA protein does not induce membrane vesiculation. It appears that MxA protein-linked GTP hydrolysis influences flow dynamics between sub-compartments of the smooth endoplasmic reticulum (Accola *et al.*, 2002, Stertz *et al.*, 2006).

2.5.2. MxA protein is involved in Ca2 +/-dependent signalling

Many cellular functions are regulated by the intracellular Ca^{++} concentration such as cell growth, differentiation, concentration or secretion. The transient receptor potential canonical (TRPC) subfamily consists of calcium permeable cation channels, which are involved in the regulation mechanisms of intracellular Ca^{++} (Birnbaumer *et al.*, 2000, Vennekens *et al.*, 2002). An increase in the intracellular calcium concentration is in response to a phospholipase C activating stimulus. This activation induces the hydrolysis of phosphatidylinositol 4,5-biphosphate to two secondary messengers, in particular 1,4,5-triphosphate (IP3) and diacylglycerol. IP3 activates the receptor at the level of the endoplasmic reticulum and induces a first phase of increase of Ca^{++}, which is the consequence of a release of calcium from the intracellular pool. The second phase of intracellular Ca^{2+} increase involves the re-entry of calcium from the extracellular pool (Lussier *et al.*, 2005). Functional TRPC complexes are both homo- and heterotetramers and each TRPC subunit has domains that typically interact with other proteins (Lussier *et al.*, 2005).

These TRPC subunits have, at their N-terminus, so-called "coiled coil" regions and a hyperconserved region composed of three or four ARDs (ankyrin-like repeat domains) (Vazquez *et al.*, 2004). It has been demonstrated that the C-terminus of MxA protein interacts not only with the second ARD domain of TRPC1, -3, -4, -5, -6, and -7, but also stimulates the activity of TRPC-1. 6 (Lussier *et al.*, 2005). GTP binding to MxA protein appears to be important for modulation of TRPC6 activity, but not GTPase activity (Lussier *et al.*, 2005). Thus, it cannot exclude an implication of MxA protein in TRPC-dependent cell trafficking. Therefore, MxA protein could be considered as a new regulatory protein involved in calcium-dependent signaling. However, further studies will be needed to define the exact mechanism of action (Lussier *et al.*, 2005).

2.5.3. MxA protein is involved in motility

MxA protein adheres to tubulin, inhibits cell motility in vitro and metastatic potential in vivo. The PC-3M clone of the PC-3 human prostate carcinoma line is extremely aggressive. This line has a chromosomal lesion that caused the excision of the genes encoding MxA protein. When exogenous MxA is expressed in this PC-3M clone, its motility and invasiveness collapse in vitro. On the other hand, when this clone expresses MxA protein stably, the number of hepatic metastases decreases significantly after intrasplenic injection. In addition, overexpression of exogenous MxA protein in malignant melanoma cells also inhibits mobility and invasiveness. In coimmunoprecipitation, a MxA / tubulin association has been demonstrated and this molecular link is related to the effect of MxA protein on motility and invasiveness (Mushinski *et al.*, 2009).

2.5.4. Mx proteins - GTPases with anti-viral activity

Although several roles have been associated with human MxA protein, the only Mx-dependent function widely accepted by the entire scientific community is its antiviral activity. Here, it should be

noted that only some isoforms exert an antiviral activity. The exact mechanism of action is still unclear, but several strong hypotheses are advanced. Schematically, the authors describe / postulate an interaction between certain viral proteins and homo-oligomers Mx (Kochs and Haller, 1999a, Haller and Kochs, 2002) or a direct interaction between active Mx proteins and certain cellular structures, the latter hypothesis having been emitted following the observation of cell-type-dependent antiviral activity expressing the studied Mx protein (Meier *et al.*, 1990, Schneider-Schaulies *et al.*, 1994, Landis *et al.*, 1998).

The antiviral activity of a Mx protein has been described for the first time by the discovery of a resistance phenotype: some strains of laboratory mice survive typically deadly doses of murine influenza A virus for other strains of mouse. Afterwards, numerous studies have been published describing the antiviral activity of a given Mx protein against one or the other virus. Taken together, these studies suggest a typical profile for Mx-susceptible viruses: having a genome made up of a single strand of negative polarity RNA. Thus, there may be mentioned an antiviral activity of the Mx protein against the family (a) of *Orthomyxoviridae* (*Influenza A, Thogoto virus, Dhori and Batkenvirus*) (Pavlovic *et al.*, 1990, Haller *et al.*, 1995, Frese *et al.*, 1997), (b) *Rhabdoviridae* (VSV virus [vesicular stomatitis]) (Schwemmle *et al.*, 1995b, Pavlovic *et al.*, 1990), (c) *Paramyxoviridae* (*measles virus* and *parainfluenza human virus type 3*) (Schnorr *et al.*, 1993, Schneider-Schaulies et al., 1994, Zhao *et al.*, 1996), and (d) *Phenuiviridae* (*fever virus of the Rift, Lacrosse virus, Dugbe virus, Crimean-Congo haemorrhagic fever virus, Hantaan virus, Puumula-and Tulavirus*) (Hefti *et al.*, 1999, Frese *et al.*, 1996, Kanerva *et al.*, 1996, Kochs *et al.*, 2002b, Andersson *et al.*, 2004, Bridgen *et al.*, 2004). However, recently this standard profile has tended to fade since the antiviral activity of Mx proteins has been shown to affect other families of viruses, for example (a) *Togaviridae* (*Semliki Forest virus*) (Hefti *et al.*, 1999, Landis *et al.*, 1998), (b) *Picornaviridae* (Coxsackievirus B4) (Chieux *et al.*, 2001), (c) *Hepadnaviridae* (*hepatitis B virus*) (Gordien *et al.*, 2001b) and (d) *Birnaviridae* (infectious pancreatic necrosis virus) (Larsen *et al.*).

Before discussing the inventory of Mx isoforms having demonstrated antiviral activity, the reader will find in Table 3 a census of the different families of viruses in which at least one member is inhibited by at least one Mx protein.

Table 3: The basic biological characteristics of common virus families.

Virus	Genome	Cellular entrance	Place of propagation	References
Orthomyxoviridae	Single-stranded RNA, segmented of negative polarity	1.Endocytosis dependent on clathrin 2. Caveolin-dependent endocytosis 3.Endocytosis dependent on clathrin and caveolin	Cytoplasm/ nuclear	(Sieczkarski and Whittaker, 2003, Nunes-Correia *et al.*, 2004, Neumann *et al.*, 2004, Sieczkarski and Whittaker, 2002)
Rhabdoviridae	Single-stranded RNA, unsegmented of negative polarity	1.Endocytosis dependent on clathrin	Cytoplasm	(Banerjee, 1987, Sieczkarski and Whittaker, 2003, White *et al.*, 1981, Das *et al.*, 2006, Matlin *et al.*, 1982)
Paramyxoviridae	Single-stranded RNA, unsegmented of negative polarity	1. Caveolin-dependent endocytosis 2. Direct fusion with the plasma membrane	Cytoplasm	(Cantin *et al.*, 2007, Palermo *et al.*, 2007)
Phenuiviridae	Single-stranded RNA, segmented of negative polarity	1.Endocytosis dependent on clathrin	Cytoplasm	(Bouloy, 1991, Jin *et al.*, 2002)
Togaviridae	Single-stranded RNA, linear, unsegmented, positive polarity	1.Endocytosis dependent on clathrin	Cytoplasm	(Kuhn, 2007, Sieczkarski and Whittaker, 2003).
Picornaviridae	Single-stranded RNA, linear, unsegmented, positive polarity	1.Endocytosis dependent on clathrin 2.Dependent internalisation of lipid-rafts	Cytoplasm	(Triantafilou and Triantafilou, 2003, Triantafilou and Triantafilou, 2004, Laude and Prior, 2004, Chung *et al.*, 2005).
Hepadnaviridae	Partially double-stranded, circular, non-segmented DNA	1.Endocytosis dependent on clathrin	Cytoplasm/ nuclear	(Funk *et al.*, 2006, Breiner and Schaller, 2000).
Birnaviridae	Segmented double-stranded RNA	1.Endocytosis dependent on clathrin	Cytoplasm	(Espinoza and Kuznar, 1997)

* Mouse Mx1 and Mx2 proteins:

Mouse Mx1 protein was discovered due to its anti-influenza activity (Lindenmann, 1962). Later, crosses between the virus resistant mouse strains and susceptible mouse strains establish the important role of the Mx protein for this resistance phenotype. Then, after the establishment of appropriate cell lines, it has been demonstrated, in vitro, that the mouse Mx1 protein alone is capable of inhibiting the multiplication of influenza A viruses. In addition, the mouse Mx1 protein also has antiviral activity against some other members of the *Orthomyxoviridae* family such as: (a) *Thogoto virus* (Thimme *et al.*, 1995), (b) *Dhori virus* (Frese *et al.*, 1997), and (c) *Batken virus* (Jin *et al.*, 1999). In contrast, murine Mx1 protein has no antiviral activity against viruses of other families. Among the inbred strains of laboratory mice, a mutation responsible for a shift in the reading frame induces the expression of a non-functional Mx2 protein. However, in some strains of wild mice, a functional Mx2 protein is expressed in the cytoplasm; this inhibits the multiplication of *vesicular stomatitis virus* (Zürcher *et al.*, 1992, Jin *et al.*, 2001) and *Hantaan virus* (Arnheiter and Haller).

* Human MxA and MxB proteins:

Human MxA protein has been found to inhibit many viruses from different families meanwhile the MxB protein has no antiviral activity (Pavlovic *et al.*, 1990). The establishment of constitutive expression of MxA protein has demonstrated antiviral activity against *influenza A virus* (Pavlovic et al., 1990, Frese *et al.*, 1995), *Thogoto virus* (Frese *et al.*, 1995) and *influenza C viruses* (Marschall *et al.*, 2000). MxA protein also inhibits the multiplication of *VSV*, a member of the *Rhabdoviridae* family (Schwemmle *et al.*, 1995b, Schnorr *et al.*, 1993, Staeheli and Pavlovic, 1991). In *Phenuiviridae* family, the human MxA protein inhibits the multiplication of *La Crosse virus* (Frese et al., 1996, Miura *et al.*, 2001), *Hantaan, Tula and Puumala viruses* (Frese *et al.*, 1996, Kanerva *et al.*, 1996), *Rift Valley fever virus, sandfly fever* (Frese *et al.*, 1996), *Dugbe virus* (Bridgen *et al.*, 2004) and *Crimean-Congo haemorrhagic fever virus* (Andersson *et al.*, 2004). Some members of the *Paramyxoviridae* family and *Togaviridae* family (Semliki Forest virus) are inhibited in more specific cell preparations; therefore, the inhibition of virus multiplication may depend on the type of cells (Zhao *et al.*, 1996, Schnorr *et al.*, 1993, Schneider-Schaulies *et al.*, 1994, Pavlovic *et al.*, 1990, Landis *et al.*, 1998). Recently, an antiviral activity of MxA protein against *Coxsackievirus B4* has been described (*Picornaviridae* family) (Chieux *et al.*, 2001); this is a particular observation since, simultaneously, two other members of the same family are insensitive to the antiviral activity of MxA protein (Pavlovic *et al.*, 1990). The MxB protein also has activity against a virus with a genome of the DNA type, it is the *Hepatitis B virus* (*Hepadnaviridae* family) (Gordien et al., 2001b, Peltekian *et al.*, 2005).

Table 4: Antiviral Activities of Human MxA protein.

Size (aa)	Subcellular Localization	Proven antiviral activity Virus	Family/genre	References
661	Cytoplasm	Influenza A	Orthomyxoviridae Influenzavirus A	(Pavlovic *et al.*, 1990, Frese *et al.*, 1995).
		Influenza B	Orthomyxoviridae Influenzavirus B	(Marschall *et al.*, 2000)
		Thogotovirus	Orthomyxoviridae Thogotovirus	(Pavlovic *et al.*, 1995, Frese et al., 1996).
		VSV	Rhabdoviridae Vesiculovirus	(Frese *et al.*, 1996, Pavlovic *et al.*, 1990).
		La Crosse virus	Phenuiviridae Bunyavirus	(Frese *et al.*, 1996, Miura *et al.*, 2001).
		Hantaan virus	Phenuiviridae Hantavirus	(Frese *et al.*, 1996)
		Puumala virus	Phenuiviridae Hantavirus	(Kanerva *et al.*, 1996)
		Tula virus	Phenuiviridae Hantavirus	(Frese *et al.*, 1996)
		Rift Valley fever virus	Phenuiviridae Phlebovirus	(Frese *et al.*, 1996)
		Sand fly fever virus	Phenuiviridae Phlebovirus	(Frese *et al.*, 1996)
		Dugbe virus	Phenuiviridae Nairovirus	(Bridgen *et al.*, 2004)
		Crimean-Congo hemorrhagic fever virus	Phenuiviridae Nairovirus	(Andersson *et al.*, 2004)
		Human Parainfluenza Type 3	Paramyxoviridae Respirovirus	(Zhao *et al.*, 1996, Atreya and Kulkarni, 1999)
		Measles virus	Paramyxoviridae Morbillivirus	(Schnorr *et al.*, 1993, Schneider-Schaulies *et al.*, 1994)
		Semliki Forest virus	Togaviridae Alphavirus	(Hefti *et al.*, 1999, Landis *et al.*, 1998)
		Coxsackievirus B4	Picornaviridae Entérovirus	(Chieux *et al.*, 2001)
		Virus de l'Hépatite B	Hepadnaviridae Orthohepadnavirus	(Gordien *et al.*, 2001a)

* Chicken Mx1 protein:

The chicken Mx1 protein is pleomorphic (numerous allelic variations). Different isoforms are able to limit the multiplication of VSV and influenza A (Ko *et al.*, 2002).

* Porcine Mx1 protein:

By analyzing the exon 14 sequence of the porcine Mx1 protein, different isoforms have been identified (ASANO *et al.*, 2002), among which, two isoforms were tested with their antiviral activity and they were found to inhibit the multiplication cycle of VSV; however, concerning the inhibition of

the multiplication of influenza A viruses, one of the two isoforms demonstrates greater antiviral activity than the other (Palm *et al.*, 2007).

* Cattle Mx1 and Mx2 proteins:

The inducible expression of the bovine Mx1 protein in Vero cells has demonstrated an inhibitory activity against *VSV* (Baise *et al.*, 2004). In the presence of the bovine Mx1 protein, there is also a reduction in the production of infectious particles of the *rabies virus* (*Rhabdoviridae* family) (Leroy *et al.*, 2006a). More recently, it has been shown that bovine Mx2 protein exhibits antiviral activity against VSV (Babiker *et al.*, 2007), but not against influenza A viruses.

* Rat Mx1, Mx2 and Mx3 proteins:

In the rat, three Mx proteins are expressed including Mx1, Mx2 and Mx3. The Mx1 protein exerts antiviral activity against *influenza A viruses*, *Thogoto virus* (Sandrock *et al.*, 2001) and *VSV* (Meier *et al.*, 1990). The Mx2 protein inhibits the multiplication of *VSV* as well as some other members of *Phenuiviridae* family such as *Rift Valley fever virus* and *La Crosse virus* (Sandrock *et al.*, 2001).

Table 5: Antiviral activities of rodent Mx1 proteins.

Mx	Size (aa)	Subcellular localization	Proven antiviral activity Virus	Family	References
Mx1 murine	631	Noyau	Influenza A	Orthomyxoviridae Influenzavirus A	(Staeheli *et al.*, 1986b, PAVLOVIC and STAEHELI, 1991)
			Thogotovirus	Orthomyxoviridae Thogotovirus	(Haller *et al.*, 1995)
			Dhori virus	Orthomyxoviridae Thogotovirus	(Thimme *et al.*, 1995)
			Batken virus	Orthomyxoviridae Thogotovirus	(Frese *et al.*, 1997)
Mx2 murine	655	Cytoplasm	VSV	Rhabdoviridae Vesiculovirus	(Jin *et al.*, 1999, Zürcher *et al.*, 1992)
			Virus Hantaan	Phenuiviridae Hantavirus	(Jin *et al.*, 2001)
Mx1 rat	652	Nuclear	Influenza A	Orthomyxoviridae Influenzavirus A	(Meier *et al.*, 1990)
			Thogotovirus	Orthomyxoviridae Thogotovirus	
			VSV	Rhabdoviridae Vesiculovirus	(Meier *et al.*, 1990)
Mx2 rat	659	Cytoplasm	VSV	Rhabdoviridae Vesiculovirus	(Meier *et al.*, 1990)
			Rift Valley fever virus	Phenuiviridae Phlebovirus	(Sandrock *et al.*, 2001)
			La Crosse virus	Phenuiviridae Bunyavirus	(Sandrock *et al.*, 2001)

Table 6: Antiviral Activities of other Mx1 proteins.

Mx	Size (a.a)	Subcellular localisation	Proven antiviral activity Virus	Family	Reference
Poultry Mx1	705	Cytoplasm	Influenza A	Orthomyxoviridae Influenzavirus A	(Ko *et al.*, 2002)
			VSV	Rhabdoviridae Vesiculovirus	(Ko *et al.*, 2002)
Porcine Mx1	663	Cytoplasm	VSV	Rhabdoviridae Vesiculovirus	(ASANO *et al.*, 2002)
			Influenza A	Orthomyxoviridae Influenzavirus A	(Palm *et al.*, 2007)
Canine Mx2	711	Cytoplasm	VSV	Rhabdoviridae Vesiculovirus	(Nakamura *et al.*, 2005)
Bovine Mx1	654	Cytoplasm	VSV	Rhabdoviridae Vesiculovirus	(Baise *et al.*, 2004)
			Virus de la rage	Rhabdoviridae Lyssavirus	(Leroy *et al.*, 2006b)
Bovine Mx2	710	Cytoplasm	VSV	Rhabdoviridae Vesiculovirus	(Leroy et al., 2006b)

► Canine Mx1 and Mx2 proteins

When expressed, the Mx1 and Mx2 canine proteins are located in the cytoplasm. Stable expression of Mx2 protein in mouse 3T3 fibroblasts confers anti-VSV activity, whereas expression of Mx1 protein confers no antiviral activity (Nakamura *et al.*, 2005). In Tables 4, 5 and 6, the reader will find a synopsis of the antiviral activities of the different Mx isoforms studied so far, with the most relevant references.

2.6. Molecular mechanisms underlying antiviral function

2.6.1. Influenza viruses

The murine Mx1 is a nuclear protein, which is expressed as the murine Mx1 gene and is stimulated with type I interferons (Dreiding *et al.*, 1985). The protein is a high molecular weight GTPase that alone confers selective resistance against influenza A viruses (PAVLOVIC and STAEHELI, 1991, Pavlovic et al., 1990, Staeheli et al., 1986b). For the replication of its genome, influenza A viruses depend on the de novo synthesis of proteins; thus, in infected host cells exhibiting a block in protein synthesis, the replication cycle is abortive, i.e. stopped after primary transcription (Pavlovic *et al.*, 1992). Various aspects of the molecular mechanism underlying the anti-influenza activity of mouse Mx1 protein are elucidated. Thus, we know that the Mx1 protein does not interfere with the penetration of the virus into the host cell, nor with the uncoating of the virus, nor with the transport of parental nucleocapsids to the nucleus of the host cell (Horisberger *et al.*, 1980, Meyer and Horisberger, 1984). However, it has been shown that mouse Mx1 protein interferes with the accumulation rate of primary transcripts; in addition, we know that the degree of inhibition is

correlated with the length of the genomic segment. Thus, it appears that the expression of the mouse Mx1 protein interferes with elongation rather than transcription initiation (Pavlovic *et al.*, 1992). In fact, the inhibitory activity of the Mx1 protein on the multiplication cycle of the influenza A viruses can be neutralized by the overexpression of the three PB1, PB2 and PA proteins (constituting the viral polymerase); to a lesser extent, overexpression of PB2 alone reproduces the same neutralizing effect (Huang *et al.*, 1992, Stranden *et al.*, 1993). Nevertheless, a direct interaction between the mouse Mx1 protein and the PB2 polymerase subunit is not yet described (Huang *et al.*, 1992, Stranden *et al.*, 1993). These observations make it possible to hypothesize either a physical interaction or a functional competition between the mouse Mx1 protein and certain components of the viral polymeric complex, thereby blocking its normal functioning (Pavlovic *et al.*, 1992).

The human MxA protein: regarding the molecular mechanism underlying the anti-influenza activity of the human MxA protein, certain aspects are elucidated. Thus, it is known that in vivo infected host cells expressing MxA protein, (a) the accumulation rate of primary viral transcripts is not affected, (b) the primary viral transcripts are polyadenylated and adequately direct the synthesis of proteins and (c) primary viral transcripts were accumulated in the cytoplasm (Zurcher *et al.*, 1992). Nevertheless, it has been shown that in infected host cells expressing MxA protein, there is a decrease in the rate of accumulation of total viral RNA, compared with cells not expressing MxA (Zurcher *et al.*, 1992). Thus, it is assumed that MxA protein affects either (a) viral mRNA access to protein synthesis machinery, or (b) synthesis of viral proteins, or (c) transport of newly synthesized viral proteins to the nucleus or (d) replication of the viral genome. For further guidance on the exact molecular mechanism of human MxA protein, a nuclear accumulation MxA protein has been constructed (Turan *et al.*, 2004, Zurcher et al., 1992). This nuclear protein inhibits the transcription of the viral genome in the same way as the mouse Mx1 protein (Turan *et al.*, 2004); despite this similarity, the overexpression of PB2 only to a small extent neutralises the anti-influenza activity exerted by the nuclear MxA protein. However, overexpression of the viral nucleoprotein (NP) is found to be more effective (Turan *et al.*, 2004); in addition, it has been demonstrated that both nuclear MxA protein and cytoplasmic MxA protein associate with NP. Thus, it can be assumed that the nuclear MxA protein interferes with viral transcription either directly or indirectly through interaction with the viral NP protein (Turan *et al.*, 2004). Finally, it is known that NP is an essential factor for the elongation of RNA chains; as a result, the nuclear MxA protein could inhibit the elongation of the RNA chain (Honda *et al.*, 1988, Frese et al., 1995).

On the other hand, the RNA helicase UAP56, known for its role as "chaperone" in the binding of NP to viral RNAs, has been proposed as a target for MxA protein to explain the antiviral effect of this protein (Wisskirchen *et al.*, 2011).

2.6.2. The thogoto virus

The human MxA protein: *Thogoto virus* was the most sensitive to the anti-viral activity of MxA protein (Kochs and Haller, 1999a). MxA protein recognizes the incoming nucleocapsids of the *Thogoto virus* (Kochs and Haller, 1999b, Weber *et al.*, 2000) and blocks their centripetal trafficking to the nucleus of the host cell. The molecular mechanism responsible for this transport inhibition has not yet been fully elucidated (Kochs and Haller, 1999b). Several hypotheses are put forward: (a) MxA multimers would position themselves around the nucleocapsid and thus hide the nuclear localization signal of the incoming nucleocapsids or (b) MxA protein would direct the incoming nucleocapsid to compartments of the cytoplasm where the MxA- nucleocapsid would be immobilized and degraded (Pavlovic *et al.*, 1990).

2.6.3. The vesicular stomatitis virus

The human MxA protein: murine 3T3 cells constitutively expressing human MxA protein acquire strong resistance against VSV (Staeheli and Pavlovic, 1991). In these cells, the primary transcripts of the viral genes are not accumulated; it appears that MxA protein interacts with mRNA synthesis either by altering the activity of the RNA polymerase complex or by indirectly destabilizing the viral mRNAs. However, viral mRNAs obtained from cells expressing MxA protein or from control cells bind to oligod (T) in a similar manner, demonstrating the absence of intervention on polyadenylation of transcripts (Staeheli and Pavlovic, 1991). The inhibitory effect of MxA protein on primary VSV transcripts is inversely proportional to: (a) the order of the genes on the viral (linear) genome and (b) the transcriptional activity of the different genes. The "gradient" inhibitory activity of MxA could reflect direct inhibition of the viral polymeric complex. In this case, the hypothesis that MxA protein would affect the process of elongation of the RNA chain without affecting the initiation process holds up (Schwemmle *et al.*, 1995b). Nevertheless, as the purified MxA protein has been shown to be able to inhibit the transcriptional activity of VSV vRNPs, it does seem that cellular factors of the host do not interfere with the inhibition process (Staeheli and Pavlovic, 1991). In cells expressing MxA protein and previously treated with cycloheximide, the concentration of parental genomic RNA is not affected, suggesting that MxA protein does not intervene on a step before the uncoating (Frese *et al.*, 1996).

2.6.4. The La Crosse virus

Expressed by cultured cells or transgenic mice, MxA protein inhibits the multiplication cycle of *La Crosse virus* (LACV) (Hefti et al., 1999, Miura et al., 2001, Hacker *et al.*, 1989). LACV has a single-stranded RNA genome of negative polarity. The virus transcribes and replicates its genome exclusively in the cytoplasm of the host cell. Primary transcription and translation are catalyzed by viral RNA polymerase. Switching from transcription mode to replication mode requires the neosynthesis of N viral proteins. Therefore, when the concentration of unassembled N proteins is

limited, it interferes with the replication of the LACV genome (Frese *et al.*, 1996). In cells expressing MxA protein and infected with LACV, accumulation of primary viral transcripts does not appear to be affected; however, the amplification of the viral genome is significantly decreased (Kochs *et al.*, 2002b). These phenomena are consistent with observations that MxA protein recognizes the incoming LACV N protein and that, subsequently, MxA protein interacts with the N protein to form elongated tubular structures giving rise to perinuclear complexes (Kochs *et al.*, 2002b). Indeed, these observations suggest that MxA "sequesters" the free N protein, which makes it less available for its functions in the viral cycle, including the switch transcription/replication. The ultimate fate of MxA/N complexes is not known (Kochs *et al.*, 2002b). Finally, MxA protein interferes in the same way with the replication cycle of other Bunyamwera viruses, such as the *Rift Valley fever virus* or the *Crimean-Congo haemorrhagic fever virus* (Andersson *et al.*, 2004).

2.6.5. The measles virus

The human MxA protein expressed in a monocyte line. In case of acute measles, the virus replicates in monocytes and alters its functions (Hyypiä *et al.*, 1985, Osunkoya *et al.*, 1974, Wrzos *et al.*, 1979, McChesney and Oldstone, 1987). In monocytes expressing MxA protein, the transcription of the measles virus is unaffected, the synthesis and stability of the corresponding mRNAs either. However, in monocytes the expression of MxA protein inhibits the expression of the so-called "structural" proteins of the virus. Therefore, it is believed that the anti-viral activity of MxA protein occurs at a time after transcription (Schnorr *et al.*, 1993).

Human MxA protein expressed in brain cells. Chronic infection of the central nervous system with the measles virus is characterized by an accumulation of viral ribonucleoproteins in neurons and glial cells. In addition, under these conditions, there is a sharp decrease in the level of expression of proteins in the viral envelope (Schneider-Schaulies and ter Meulen, 1992, Kraus *et al.*, 1992). Neurons infected with the measles virus show significant expression of MxA protein (Schneider-Schaulies *et al.*, 1994); here, this protein is capable of conferring resistance against viral infections (Schneider-Schaulies *et al.*, 1994). In human glia, the expression of MxA protein causes a decrease in the efficiency of transcription as evidenced by the reduction in the amounts of viral mRNA.

Thus, within monocytes, MxA protein causes a decrease in the expression of viral glycoproteins via a transcriptional blockade whereas in glial cells, the expression of MxA protein induces a decrease in the production of the internal proteins "via a translational blockade" (Schneider-Schaulies *et al.*, 1994)s.

2.6.6. The semliki forest virus

The human MxA protein: *Semliki Forest* virus (SFV) (*Togaviridae*) is a single-stranded RNA virus of positive polarity. This virus enters its host cell via endocytosis via an interposed receptor (Marsh and Helenius, 1989) and then undergoes an uncoating process that releases the nucleocapsid (Singh and Helenius, 1992). Virus replication takes place in the cytoplasm of the infected cell and the released viral genome directly serves as mRNA. It would appear that the activity of MxA protein against SFV depends on either the species or the cell type (Landis *et al.*, 1998). This dependence suggests the need for the presence of a cellular factor (Landis *et al.*, 1998). In cells expressing MxA protein and infected with SFV, there is a reduction in: (a) viral protein synthesis, (b) viral mRNA transcription and (c) genome amplification (Landis *et al.*, 1998). It is therefore conceivable that MxA protein interacts with an early stage of the SFV cell cycle. Thus, the targets for the inhibitory activity of MxA protein could be: (a) the synthesis or the functioning of the viral replicase, (b) the synthesis or the elaboration of the structural proteins, (c) the replication of the Genomic viral RNA, or (d) synthesis or capping of viral RNA. It is also possible that it is an interaction of MxA protein with one or more cellular proteins which causes the blockage (Landis *et al.*, 1998).

2.7. Pathological expression of Mx proteins

2.7.1. Overexpression of MxA protein in Fanconi anaemia

Fanconi anaemia results from an autosomal and recessive genetic defect. Dissection of the genetic bases underlying this clinical syndrome led to the identification of 5 different subtypes; these share some clinical and cellular features (Duckworth-Rysiecki *et al.*, 1985, Strathdee *et al.*, 1992, Joenje *et al.*, 1995). Affected individuals have skeletal and visceral malformations, a predisposition for cancer development, and a predisposition for bone marrow failure as well as acute leukaemia (Liu *et al.*, 1994). In 1997, Li and Youssoufian showed that in four of the five subtypes, patients showed overexpression of MxA protein in the absence of any exogenous interferon-dependent stimulation (Li and Youssoufian, 1997). The overexpression of MxA protein probably induces early apoptosis, but no mechanistic link has yet been established between this overexpression and either symptom or lesion.

2.7.2. Overexpression of MxA protein in alopecia areata

Alopecia areata (AA) is an autoimmune disease characterized by incomplete hair loss and infiltration of hair follicles by T cells. People with Down syndrome (DS, mongolism) exhibit a high frequency of AA superior to the general population. DS is linked to an additional copy (whole or partial) of chromosome 21 (Tazi-Ahnini *et al.*, 2000); this chromosomal region could therefore carry the genes involved in the pathogenesis of AA (Reeves *et al.*, 1988). The human MxA gene is located within a region of chromosome 21, including the minimum triplicate fragment. According to some authors, MxA protein is therefore probably involved in the manifestations of Down syndrome (Tazi-Ahnini *et al.*, 2000). On the other hand, within the abnormal hair follicles of people with AA, (and not

in their normal follicles) MxA protein is highly overexpressed. Again, some authors establish a causal link. When MxA gene in AA patients was sequenced, 4 particular SNPs (single nucleotide polymorphism) were identified in intron 6. An association study subsequently confirmed a significant association between this genetic marker and patients with AA (Tazi-Ahnini *et al.*, 2000).

2.7.3. Overexpression of MxA Protein in Subacute Sclerosing Panencephalitis

Panencephalitis Subacute sclerosing panencephalitis (SSPE) is a neurodegenerative disease that results from chronic slow virus infection by a mutated measles virus (Gascon, 1996); this disease is found in children and young adults. MxA protein is known to inhibit the multiplication of certain single-stranded RNA viruses, including the multiplication of measles virus (Schneider-Schaulies *et al.*, 1994, Schnorr *et al.*, 1993). Since two particular SNPs within the human MxA gene promoter region is associated with SSPE, some authors hypothesize a genetic predisposition. Mechanistically, the same authors even suggest a causal link between a dysregulation of MxA expression and the establishment of persistent infection with the measles virus (Torisu *et al.*, 2004). This hypothesis remains a speculation to date for lack of work dedicated to its experimental verification.

CHAPTER II: OBJECTIVES

Since their discovery in 1962, Mx proteins have been the subject of many scientific studies. All relate to one or other of the following two themes: (1) its use as a biomarker of the secretion of type I and type III interferons and (2) its role in innate immunity against viral infections. My PhD dissertation does not derogate from this "tradition".

Water buffalo *(Bubalus bubalis)* has been an integral part of livestock agriculture in Asia for over 5000 years, producing draft power, milk, meat and hides. Today, 153 million buffaloes provide about 77,000,000 tons of milk and 3,000,000 tons of meat and, in several countries, up to 30% of the draft power for agricultural operations. Unfortunately, however, the species did not receive the attention of policy and diagnostic tool makers in accordance with its socio-economical merits. To develop a clinically significant, cheap and practical enzyme-linked immunosorbent assay (ELISA) for the detection and quantitation of Mx1 protein in water buffalo whole blood, specific ligands are crucially needed. The first objective of this thesis is to contribute to the development of such diagnostic tools by generating and characterizing monoclonal antibodies and examining their usefulness in prototypic flow cytometry, immunoblotting, immunofluorescence and ELISA formats.

Type I (IFNs-α/β) and III (IFN- λ) interferons provide powerful and universal innate intracellular defense mechanisms against viruses. Among the antiviral effectors induced, Mx proteins of some species appear as key components of defense. The antiviral breadth exhibited by the most studied antiviral Mx protein so far, human MxA, is remarkable because it hinges upon detection of unique antiviral structures that differ across virus families suggesting that an antiviral Mx protein must display a platform dedicated to the recognition of specific viral targets. In order to determine the basis of MxA specificity for viral recognition, an evolution-guided approach that capitalizes on the antagonistic arms race between MxA and its viral targets on the one hand and the genomic signature it left on primate genomes on the other was recently followed. There emerged that the surface-exposed so-called “loop L4”, which protrudes from the compact structure of the MxA stalk, is a hotspot for recurrent positive selection. Further, single amino acid changes in L4 were shown to be necessary and sufficient to explain dramatic differences in species-specific antiviral activity of primate MxA proteins against orthomyxoviruses. Among the latter, influenza viruses have the ability to cause recurrent epidemics of respiratory disease in humans, poultry, pigs and horses. In these animal species, the infection is highly contagious and virtually all members of the species are repeatedly infected. Besides, bovines and buffaloes have lived in close proximity to poultry, swine, horses and humans since the beginning of their domestication, i.e. in direct contact with these most contagious species. Their exposure and sensitivity to influenza virus infection is attested by both serological surveys and experimental inoculation trials. Conversely, the canine species seems to have been exposed to influenza viruses only recently. Prior to 2004, only sporadic outbreaks of canine influenza had been

observed in dog populations around the world. In 2004, however, an H3N8 influenza virus of equine origin caused severe respiratory disease in racing greyhounds in Florida and, subsequently, cases of dogs affected with various subtypes of influenza viruses have been reported in many countries. The rationale underlying the second objective of this thesis is the following: since human MxA is archetypic of Mx1 proteins in general, it could be expected that the aforedescribed L4 loop also functions as a recognition platform for influenza viruses in the Mx1 proteins of other species that had been exposed to the virus for a long time. Accordingly, the second objective of this thesis is to quantitfy the anti-influenza activity of pig's, horse's, cow's, buffalo's and dog's Mx1 proteins in a strictly standardized experimental scheme for interpreting the results obtained with respect to their respective L4 loop.

1

CHAPTER III: EXPERIMENTAL SECTION

Anti-influenza A virus activities of type I/III interferons-induced Mx1 GTPases from different mammalian species.

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ABSTRACT

Type I/ III interferons provide powerful and universal innate intracellular defense mechanisms against viruses. Among the antiviral effectors induced, Mx proteins of some species appear as key components of defense against influenza A viruses. It is expected that such an antiviral protein must display a platform dedicated to the recognition of said viruses. In an attempt to identify such platform in human MxA, an evolution-guided approach capitalizing on the antagonistic arms race between MxA and its viral targets and the genomic signature it left on primate genomes revealed that the surface-exposed so-called "loop L4", which protrudes from the compact structure of MxA stalk, is a hotspot of recurrent positive selection. Since MxA is archetypic of Mx1 proteins in general, we reasoned that the L4 loop also functions as a recognition platform for influenza viruses in the Mx1 proteins of other species that had been exposed to the virus for ever. Here, the anti-influenza activity of five distinct mammalian Mx1 proteins was measured by comparing the number of viral nucleoprotein-positive cells 7 hours after infection in a sample of 100,000 cells expected to contain both Mx1-positive and Mx1-negative cell subpopulations. The systematic depletion ($p < 0.001$) of virus nucleoprotein-positive cells among equine, bubaline, porcine and bovine Mx1-expressing cell populations compared to Mx-negative cells suggests a strong anti-influenza A activity. Looking for common anti-influenza signature elements in the sequence of these Mx proteins, we found that an aromatic residue at positions 561 or 562 in the L4 loop seems critical for the anti-influenza function and/or specificity of mammalian Mx1.

INTRODUCTION

Type I (IFN-α/β) and III (IFN-λ) interferons provide powerful and universal innate intracellular defense mechanisms against viruses. Among the antiviral effectors induced, Mx proteins of some species appear as key components of defense against influenza A viruses.

The Mx structure resembles that of other members of the dynamin-like large GTPase superfamily, consisting of a N-terminal GTPase domain (G domain) and a C-terminal stalk (Gao *et al.*, 2011). These two structural domains are linked by a bundle-signalling element that is necessary to transfer structural changes during GTP binding and hydrolysis to the stalk structure. The body of work published to date suggests that the minimum requirements of a putatively antiviral Mx protein are to possess a GTP-binding site, the structural bases allowing multimerization, and a specific C-terminal GTPase effector domain.

The antiviral breadth exhibited by the most studied antiviral Mx protein so far, human MxA, is remarkable because it hinges upon detection of unique antiviral structures that differ across virus families (Haller and Kochs, 2011). For example, differences in Mx-resistance among influenza A virus strains have been shown to depend on differences in the nucleoprotein (Zimmermann *et al.*, 2011). Furthermore, MxA activity against the alphavirus Semliki Forest virus (SFV) is independent of SFV

nucleoprotein or other structural proteins (Landis *et al.*, 1998), and MxA antiviral activity against DNA viruses like hepatitis B virus (Li *et al.*, 2012) and African swine fever virus (Netherton *et al.*, 2009) is dependent on unique viral components. These observations suggest that, in addition to the minimum structural domains required, an antiviral Mx protein must also display a platform dedicated to the recognition of specific viral targets.

In order to determine the basis of MxA specificity for viral recognition, an evolution-guided approach that capitalizes on the antagonistic arms race between MxA and its viral targets on the one hand and the genomic signature it left on primate genomes on the other was recently followed. There emerged that the surface-exposed so-called "loop L4", which protrudes from the compact structure of MxA stalk (Gao *et al.*, 2011), is a hotspot for recurrent positive selection. Further, single amino acid changes in L4 were shown to be necessary and sufficient to explain dramatic differences in species-specific antiviral activity of primate MxA proteins against orthomyxoviruses (Mitchell *et al.*, 2012).

Among the latter, influenza viruses have the ability to cause recurrent epidemics of respiratory disease in humans, poultry, pigs and horses. In these animal species, the infection is highly contagious and virtually all members of the species are repeatedly infected (Maes *et al.*, 2000, Vahlenkamp and Harder, 2006). Besides, bovines and buffaloes have lived in close proximity to poultry, swine, horses and humans since the beginning of their domestication, i.e. in direct contact with these most contagious species. Their exposure and sensitivity to influenza virus infection is attested by both serological surveys (Crawshaw *et al.*, 2008, Jones-Lang *et al.*, 1998, Lopez and Woods, 1986, Zhai *et al.*, 2017) and experimental inoculation trials (Lopez and Woods, 1987). Conversely, the canine species seems to have been exposed to influenza viruses only recently. Prior to 2004, only sporadic outbreaks of canine influenza had been observed in dog populations around the world. In 2004, however, an H3N8 influenza virus of equine origin caused severe respiratory disease in racing greyhounds in Florida and, subsequently, cases of dogs affected with various subtypes of influenza viruses have been reported in many countries (Xie *et al.*, 2016).

Since human MxA is archetypic of Mx1 proteins in general (Haller *et al.*, 2015), it is expected that the aforedescribed L4 loop also functions as a recognition platform for influenza viruses in the Mx1 proteins of other species that had been exposed to the virus for a long time. In the following study, the anti-influenza activity of pig's, horse's, cow's, buffalo's and dog's Mx1 proteins was quantified in a strictly standardized experimental scheme. The results obtained were then interpreted with respect to their respective L4 loop.

MATERIALS AND METHODS

Experimental design

The anti-influenza activity of five distinct mammalian Mx1 proteins was measured by comparing the number of viral nucleoprotein-positive cells 7 hours after infection in a sample of 100,000 cells expected to contain both Mx1-positive and Mx1-negative cell subpopulations. The operating procedure ensured that all cells had previously been exposed to the same scenario and reagents since they had been cultured, transfected, washed, infected, fixed and stained together in the same well/tube. In addition, the absence of trypsin in the culture medium ensured that all results obtained were from the first cycle of infection. Finally, a V5 epitope flanked all the recombinant Mx1 proteins studied, which allowed an absolute standardization of the labelling of the different Mx1-positive cells to be identified by flow cytometry.

For transfection, six identical expression plasmids were used. They all encoded a single polyprotein, consisting of an RFP protein in the N-terminal position, then a 2A peptide, then a V5-Mx1 protein in the C-terminal position. Spontaneous splitting of the polyprotein into its 2 constitutive proteins was expected, with nuclear localization for the RFP protein and cytoplasmic localization for the V5-Mx1 proteins. The recombinant V5-Mx1 proteins targeted were the canine (suffix "cf", standing for *Canis familiaris*), equine ("ec", *Equus caballus*), bubaline ("bb", *Bubalus bubalis*), porcine ("ss", *Sus scrofa*) and bovine ("bt", *Bos taurus*) isoforms. In the sixth plasmid, the ORF of the canine protein contained 5 successive stop codons arranged in the N-terminal position. This plasmid was intended to exclude the possible activity of the plasmid DNA itself and/or of the RFP protein alone, the RFP-dependent fluorescence allowing identification of the cells that were duly transfected from those that had not been.

The anti-influenza activity of the above-mentioned V5-Mx1 proteins was measured with respect to 2 distinct influenza A viruses, one of mammalian origin (subtype H1N1) and the other of avian origin (H5N2). For each virus, several m.o.i. were tested and for each virus / m.o.i pair, 3 independent repeats were performed. Finally, the results reported here all come from experiments whose V5-Mx1 expression rates were comparable (between 40 and 60%).

Biochemical and biologic reagents

Cells: Human embryonic kidney cells (HEK-293T) were maintained at 37°C under a humidified atmosphere of 5% CO_2–95% air in DMEM supplemented with 10% foetal calf serum, 2 mM L-glutamine, 0.4 mM sodium pyruvate, 1x non-essential amino acids, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Plasmids: A plasmid vector (pDA657RA) was synthesized for expressing a reference canine Mx1 (NP_001003134.1) in mammalian cells. The recombinant canine Mx1 (cfMx1) intended was

fused to a N-term functional group consisting of a V5 epitope. The resulting V5-cfMx1 protein was further fused to a N-term bipartite group consisting of the fluorescent marker protein RFP followed by the spontaneously cleavable peptide 2A. In all cases, the CDS expected to encode the recombinant V5-cfMx1 protein aimed was codon-optimized, artificially synthesized by solid-phase DNA synthesis, and cloned in the said vector. Once the capacity of pDA657RA/V5-cfMx1 to drive appropriate expression of V5-cfMx1 in HEK-293T cells was duly checked, similar plasmids expressing V5 epitope-flanked equine (NP_001075961), bubaline (NP_001277782), porcine (NP_999226) and bovine (NP_776365.1) Mx1 were synthesized. Finally, 5 stop codons were introduced in the pDA657RA/V5-cfMx1 plasmid after peptide 2A, generating the control plasmid expected to express RFP alone. The 6 expression plasmids were approximately the same size (between 7901 and 7946 bp) and the sequences of all the inserts of interest were duly verified by sequencing before use. All plasmid maps are available on request.

Primary antibodies: The monoclonal anti-V5 conjugated to phycoerythrin and used in flow cytometry assays was from Abcam (ref. #ab72480) and the monoclonal anti-V5 conjugated to FITC and used in immunofluorescence studies too (ref. #ab1274). The monoclonal targeting viral NP, conjugated to FITC and used in flow cytometry studies was from Abcam (ref. #ab210526). HRP-conjugated anti-V5 tag and anti-actin monoclonal used in immunoblotting were from ThermoFisher (MA5-15253) and from Abcam (ref. ab49900), respectively.

Viruses: The influenza A viruses used were the 2 low-pathogenicity viruses A/swine/Iowa/4/76 (H1N1) and A/chicken/Belgium/150/99 (H5N2). The viruses were propagated in embryonated chicken eggs to generate the stock solutions.

Cell expansion and transfection

Thawed HEK-293T cells (ATCC® CRL-1573™) were first passaged thrice in DMEM (with 10% FCS, 1% pen-strep and 0,5% fungizone) and then seeded either in T75 cell culture flasks (immunoblotting and cytology) or onto pre-gelatinised 6-well plates ($4{,}5.10^5$ cells per well, for flow cytometry). Twenty-four hours after, cell monolayers were transfected with pDA657RA/V5-Mx1 by use of the Lipofectamine® 3000 Transfection reagent kit (ref.#L3000-008, ThermoFisher) according to the manufacturer's instructions.

Recombinant V5-Mx1 characterization by Western blotting

For immunoblotting, an 80-90% confluent monolayer in a T75 cell culture flask was transfected. Briefly, an amount of 24 µg of the plasmid DNA was diluted in P300 reagent (dilution 1:2 w/v), the mix being then added in the Lipofectamine 3000 reagent diluted in Opti-MEM medium (dilution 1:16 v/v). The final mixture was added dropwise into the T75 flask and the latter was reincubated at 37°C for 48h in a 5% CO_2 and 80% RH atmosphere. RFP-positive and –negative HEK-293T cells were sorted out by flow cytometry and resulting suspensions were washed with PBS at 4°C

and pelleted by a low-speed centrifugation. Cells pellets were lysed by boiling in Laemmli's SDS-sample buffer and reduced by mixing with NuPAGE LDS Sample Buffer (ref.#NP007, ThermoFisher Scientific) and reducing agent (ref.#NP004). Subsequently, samples were heated at 70°C for 10 min and 10 µL of each were loaded on a NuPAGE™ 4-12% Bis-Tris Protein Gels (ref. #NP0322BOX, ThermoFisher Scientific) and run at 200 V for 60 min using the XCell SureLock™ mini-cell electrophoresis system (Invitrogen). Migrated proteins were then transferred onto polyvinylidene difluoride membranes (LC2005, ThermoFisher Scientific), nonspecific binding sites were blocked with skim milk (5% in PBST) for 1h at RT and membranes were probed overnight at 4°C with an anti-V5 epitope mAb diluted in skim milk (2,5% in PBST). The blots were then washed in PBST and incubated for 1h at RT with a horseradish peroxidase-conjugated goat pAb raised against mouse IgGs (ref. #31430, diluted 1/1000 v/v, from ThermoFisher). Finally, the membranes were washed with PBST on a plate shaker for 5 min, then in deionized water for 2 min. Detection of horseradish peroxidase on membranes was finally made by use of the chemiluminescent substrate Novex® ECL. Results were visualized using the imaging system ImageQuant™ LAS 4000 and ImageJ software.

Recombinant V5-Mx1 characterization by immunocytology

For immunocytostaining, transfected HEK-293T cell suspensions were obtained as aforementioned, cytospinned and then fixed in paraformaldehyde 4% (v/v) at RT for 30 min. The fixed cells were washed with PBST, permeabilized with 0.2% (v/v) Triton X-100 for 5 min, incubated with Dako® Protein Block Serum-free (Code no. X0909) at RT for 2h, and then overnight at 4°C with 10 µg/ml of an anti-V5 epitope mAb. After washing thrice with PBST, 200 µl of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin antibody diluted 1/200 (v/v) (Abcam, code no. ab9703970) was added onto each cytospin at RT for 60 min in the dark. Excessive reagents were then removed by washing with PBST and cellular nuclei were stained with DAPI (Aldrich, code no. D9564-10MG). The slides were then thoroughly washed with PBST, mounted with Prolong Gold antifade medium (ThermoFisher Scientific, code no. P36941) and FITC/DAPI signals were examined on a Leica TCS SP5 confocal fluorescence microscope using 488nm and 405nm laser filters.

In vitro assay of Mx1 anti-influenza activity

To evaluate the antiviral activity associated with the expression of each of the targeted V5-Mx1 proteins, the percentage of influenza A virus nucleoprotein-positive cells was measured by flow cytometry in infected populations of lipofectamine-transfected HEK-293T cells. Briefly, 24h after transfection, cells were infected with 2 (H1N1) or 3 (H5N2) dilutions of the virus stock. After 1h of infection, the cells were rinsed with PBS, placed in fresh medium, cultured for another 6h, then fixed with 4% paraformaldehyde, permeabilized in 0,2% saponin and 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), blocked with 1% BSA in PBS, and probed with the corresponding primary antibodies to simultaneously detect the V5 epitope and the viral NP. The samples were finally

analysed (>100 000 cells) in an LSRFortessa flow cytometer (BD Biosciences). Instrument settings were adjusted with fresh 293T samples in 4 tubes (an unlabelled sample, 2 singly labelled samples, and 1 sample labelled with the mixed antibodies) and FACS Diva calculation software (BD Biosciences). The detection and compensation stabilities were tested before each experimental campaign with SetUp beads and dot plots were analysed with FACS DivaTM software (v.8.0.1, BD Biosciences). An example of a typical dot plot illustrating the effect of Mx1 expression on NP detection is given below :

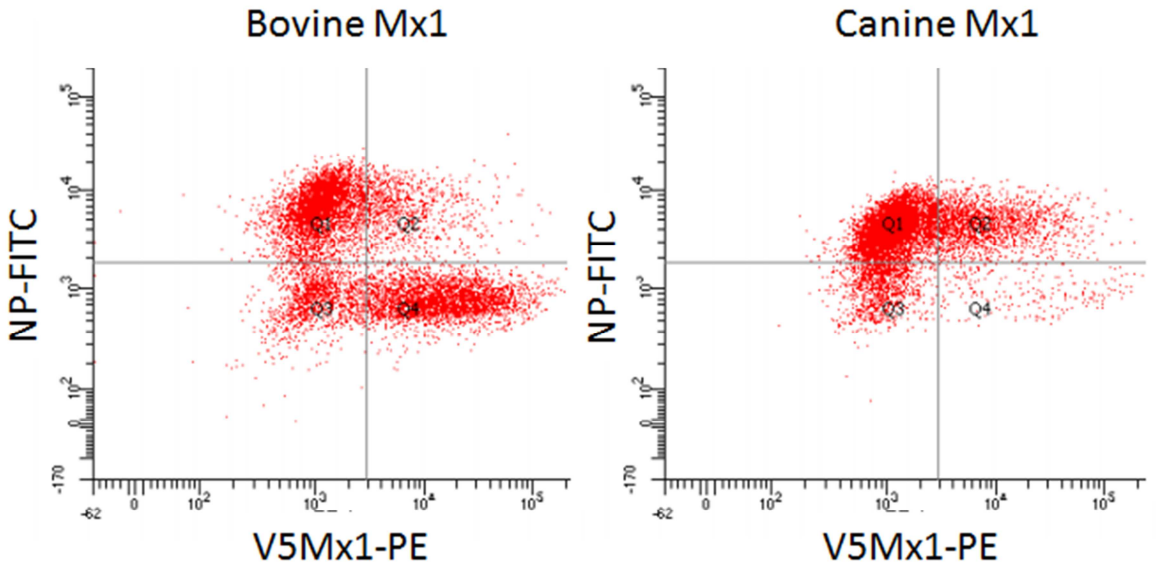


Figure 31: Typical flow cytometric dot plots showing the segregation between Mx1-positive, NP-positive and Mx1/NP–negative cells.

Data analysis

All values are presented as means ± SD. Statistical analysis was performed by analysis of variance (ANOVA) with *post hoc* testing by Fisher's protected least significant difference multiple range test.

RESULTS

Expression of recombinant V5-Mx1 proteins in HEK-293T cells

The nature and sequence of the V5-Mx1 proteins being tested were confirmed by sequencing the product of transgene-specific PCRs from transfected HEK-293T cell extracts.

Expression of recombinant V5-Mx1 proteins after transfection was assessed both qualitatively, by immunofluorescence detection of the V5 epitope in HEK-293T cells (example in Fig. 31A), and semi-quantitatively, by immunoblotting (Fig. 31B). As expected, immunocytology showed that RFP-associated fluorescence was seen in the nucleus whereas V5-Mx1 accumulated in the cytoplasm. Besides, immunoblots confirmed both the presence and the calculated molecular weight of each V5-Mx1 to be tested.

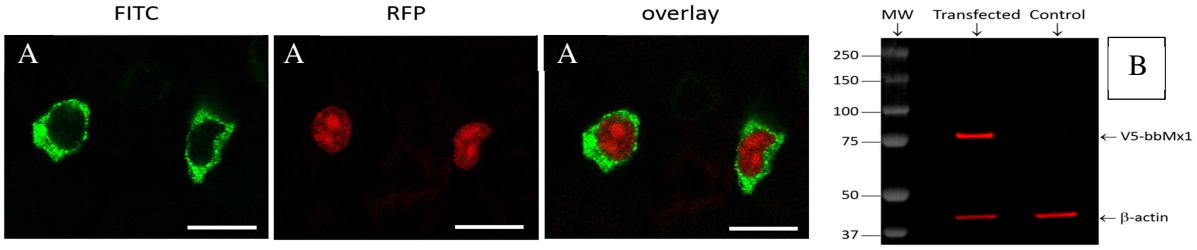


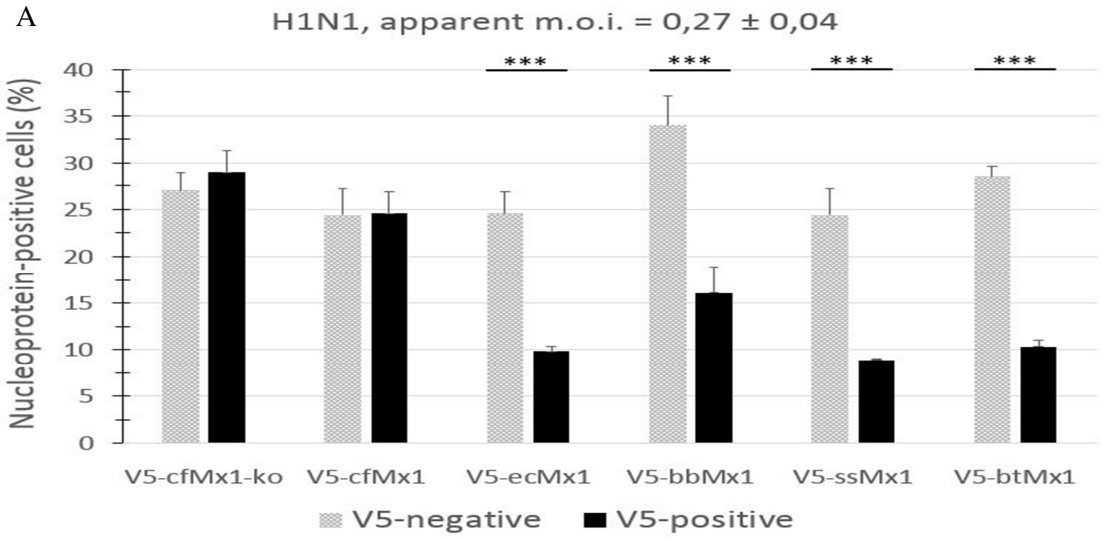
Figure 32: Expression of canine (A) and bubaline (B) V5-Mx1 in HEK-293T cells.

Notes: Two cells examined under a confocal microscope after transient transfection of plasmid pDA657RA/V5-cfMx1. Cells had been fixed 24h after transfection, permeabilized, and immunostained by incubation with monoclonal anti-V5 antibody conjugated to FITC. As expected, RFP-associated fluorescence is seen in the nucleus whereas V5-cfMx1 accumulated in the cytoplasm. B. Immunoblot of sodium dodecylsulfate 4%-12% polyacrylamide gels with total cell protein extracts from HEK-293T cells that had been transiently transfected with plasmid

Effect of V5-Mx1 proteins on influenza A virus NP synthesis

Given the reproducibility of the apparent m.o.i. (% NP-positive cells) measured among V5-negative cells within each experimental campaign (grey boxes), it is assumed that the transfection procedure and reagents equally affected all the cell populations transfected/infected (Fig. 32 & 33).

A



B

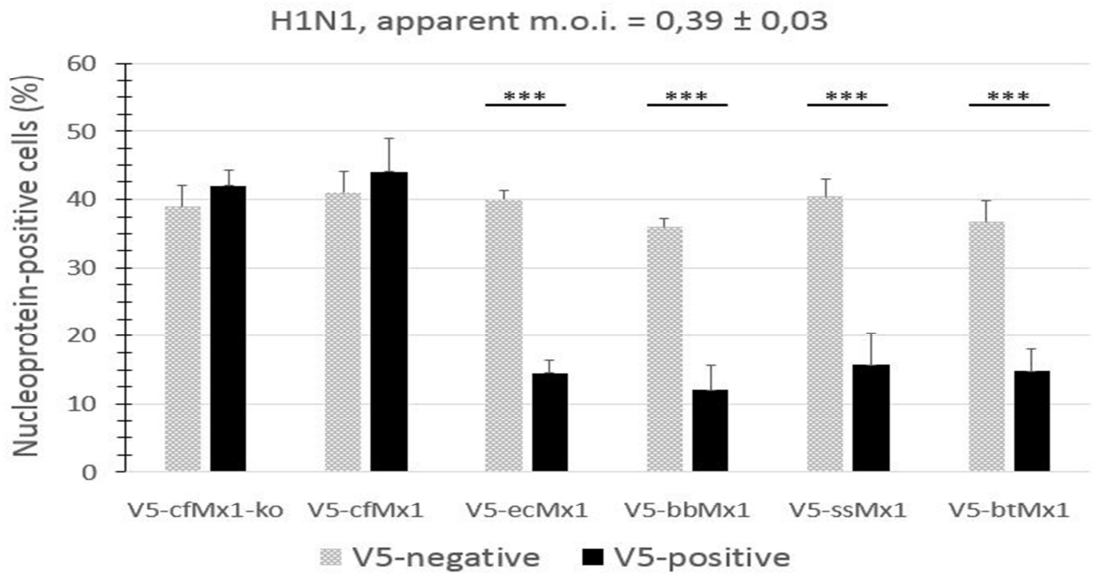


Figure 33: Percent influenza A nucleoprotein-producing cells 7h after H1N1 virus infection in recombinant Mx1 proteins non-expressing and expressing HEK-293T cell populations.

Moreover, as the infection rate of RFP-positive/V5-negative cells after transfection of expression plasmid pDA657RA/V5-cfMx1-ko tended to be similar to that of RFP-negative/V5-negative cells ($p>0.05$), it was deduced that the transduction process *per se* did not alter the viral biological cycle in the studied cell preparation. These preliminary results were valid whatever the viral load the cell monolayers were exposed to and for both influenza A strains (Fig. 32 and 33). The systematic depletion ($p < 0,001$) of NP-positive cells among equine, bubaline, porcine and bovine V5-Mx1-expressing cell populations (black boxes in Fig. 32 and 33) is therefore attributable to V5-Mx1 proteins themselves, thus suggesting a strong anti-influenza A effect at the timepoint studied. Conversely, depletion of NP-positive cells was not observed in canine V5-Mx1 expressing cells compared to the V5-negative corresponding cell population ($p>0.05$).

Comparative anti-influenza activity among different V5-Mx1 proteins

To obtain a quantitative estimate of the anti-influenza activity of each recombinant V5-Mx1 protein, we divided the apparent m.o.i. of the V5-negative cell population by that of

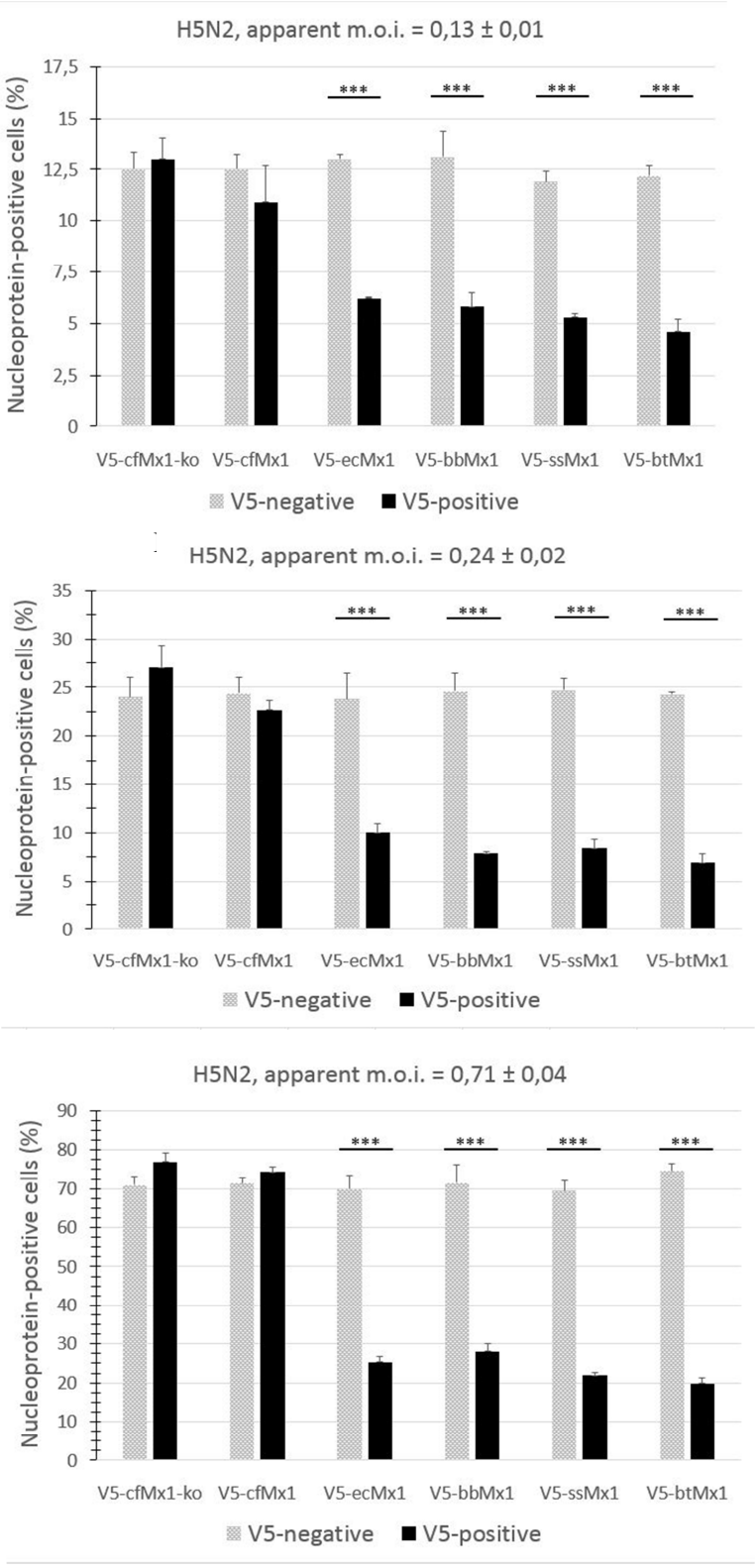


Figure 34: Percent influenza A nucleoprotein-producing cells 7 h after H5N2 virus infection in recombinant Mx1 proteins non-expressing and expressing HEK-293T cell populations.

the corresponding V5-positive population, for every experimental campaign. The results were averaged for each influenza A subtype tested (Fig. 34). As expected, the score obtained for the expression of RFP alone or for the simultaneous expression of RFP and canine V5-Mx1 is approximately 1, indicating an absence of activity. For all other V5-Mx1, the score obtained varied between 2.5 (equine V5-Mx1) and 3 (bovine V5-Mx1). Finally, for a given V5-Mx1, it is similar for the 2 viral subtypes tested ($p > 0.05$).

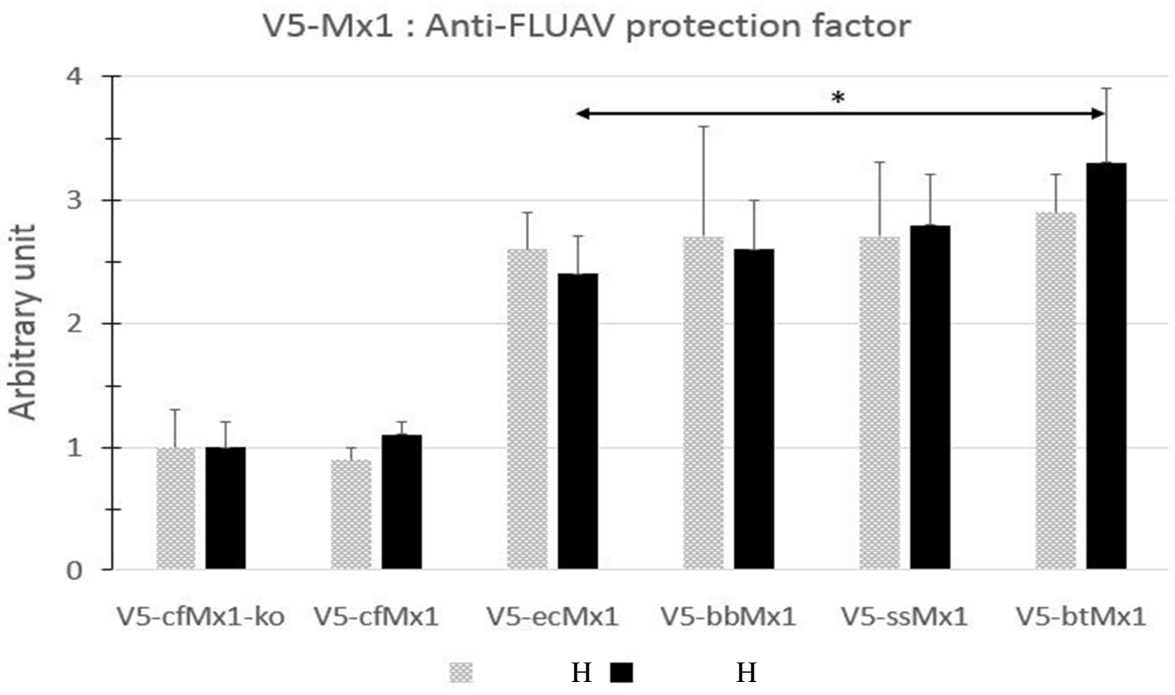


Figure 35: Quantitative estimate of the inhibition of influenza A nucleoprotein expression drived by a set of recombinant V5-Mx1 proteins.

Notes: The (%) NP-positive cells of the V5-negative cell population was divided by that of the corresponding V5-positive population, for every experimental campaign. The results were averaged for each influenza A subtype tested. The values given represents means ± SD of 2 (H1N1) or 3 (H5N2) independent campaigns.

CONCLUSIONS

1) Equine, bubaline, porcine and bovine Mx1 proteins illustrated various degrees of antiviral property against H1N1 and H5N2 influenza viruses, respectively, in a dose-dependent manner. Conversely, canine Mx1 protein did not have antiviral activity against the tested viruses.
2) For a given V5-Mx1, the antiviral activity against H1N1 was similar to that against H5N2.

1

CHAPTER IV: DISCUSSION & PERSPECTIVES

DISCUSSION

The anti-influenza function of five orthologous Mx1 proteins, all of which are localized in the cytoplasm (Fig. 31A), was examined using a highly standardized experimental design. The recombinant Mx1 proteins studied all had the expected molecular weight (Fig. 31B). The common epitope (V5) grafted in the N-terminal position made it possible to use the same mAb to detect them. The fluorescence emitted by this antibody was used as a quantitative measure of intracellular Mx1 concentration and only experimental campaigns with similar V5-specific mean fluorescence for the 5 Mx1 being tested (CV <10%) were retained for analysis. Therefore, results can be interpreted in terms of intrinsic antiviral activities of the recombinant Mx1 studied. Under the conditions of the experiment, the transfection process of the expression plasmid and the expression of the RFP protein did not cause any measurable effect on the viral cycle. Besides, an anti-influenza activity was associated with the expression of all Mx1 tested, except that of the dog.

Very little information was available about the bubaline Mx1 protein before it was tested here. An anti-rhabdovirus activity was first demonstrated after transient expression in Vero and 3T3 cells followed by manual counting of viro-positive cells over 10-20 microscopic fields (Babiker and others, 2007; Yamada and others, 2009). More recently, the relevance of the measurement of the corresponding transcript in circulating leukocytes was evaluated as a tool for the early diagnosis of pregnancy (Buragohain and others, 2016; Thakur and others, 2017). Here, it is established for the first time that bubaline Mx1 also exerts a significant anti-influenza activity since, irrespective of the viral subtype or the m.o.i., the NP-positive cell count 7h pi is decreased by about 60% (Fig 32 and 33).

Available knowledge on equine Mx1 was even rarer. At most, we know that the corresponding gene is located on chromosome 26 (Lear and others, 1998) and that two spots are detected by antisera raised against human MxA on an electrophoresis gel of total proteins extracted from equine cells exposed to IFN-α (Heinz and others, 1994). According to the results gathered here, equine Mx1 displays a significant anti-influenza activity, the strength of which matched that of bubaline Mx1 (Figs. 32 & 33).

The porcine Mx1 gene and promoter were shown to share the major structural and functional characteristics displayed by their homologs described in mouse and man (Thomas and others, 2006). In addition, allelic polymorphisms generating two very different isoforms have been identified (Nakajima and others, 2007; Palm and others, 2007). The anti-influenza activity conferred by both V5-ssMx1 isoforms was evaluated *in vitro* using either transfection of 3T3 cells followed by plaque assays (Nakajima and others, 2007) or transfection of Vero cells followed by flow cytometric

determination of the fraction of influenza virus-infected cells among Mx1-producing and -nonproducing cell populations (Palm and others, 2007). Both studies revealed that isoform α of ssMx1 is endowed with a significant anti-influenza activity. Further, this result was then consolidated by highlighting a blockade of the centripetal traffic of the incoming viral particles in the presence of ssMx1 (Palm and others, 2010). This braking of the viral cycle in the presence of ssMx1 was duly confirmed here (Figs. 32 & 33). Unexpectedly, in an elegant study dealing with mutations allowing influenza viruses to evade the antiviral function of Mx1 proteins, others concluded that the ssMx1 protein displayed a very weak anti-influenza activity (Manz and others, 2013). This result contradicts the above-mentioned experiments but also the *in vivo* observations showing that pigs are extremely resistant to experimental infections by highly virulent influenza viruses (Isoda and others, 2006). It is likely that the use of very different life cycle modeling systems (minireplicon vs. wild virus) explains these contradictory results. Discrepancy between the polymerase activity (Manz and others, 2013) and viral growth assays (this study) has indeed been observed in other studies, and is believed to result from the fact that the polymerase activity assay tests only a limited part of the influenza viral life cycle (Ashenberg and others, 2017; Herfst and others, 2010; Octaviani and others, 2011).

The antiviral spectrum of the bovine Mx1 is probably the most studied after that of human MxA. An anti-rhabdovirus activity has been detected repeatedly in several experiments conducted *in vitro*, either against VSV (Babiker and others, 2007; Baise and others, 2004; Yamada and others, 2009) or against the rabies virus (Leroy and others, 2006). On the other hand, all the paramyxoviruses tested *in vitro* so far (Sendai virus, bovine or human isolates of parainfluenza-3 virus, bovine or human strains of respiratory syncytial virus) have been shown to be bovine Mx1-resistant (Leroy and others, 2005). By contrast, *in vivo*, transgenic mice expressing bovine Mx1 are much more resistant to the mouse pneumovirus than their wild-type counterparts (Dermine and Desmecht, 2012). In an experimental design almost comparable to that used here, it had been established that the expression of an ectopic bovine Mx1 in HEK-293 cells caused a drastic decrease in the number of NP-positive cells (Garigliany and others, 2012). This result is confirmed here.

When canine Mx1 was transiently expressed in 3T3 cells, no anti-VSV activity was detected, which contrasts with canine Mx2 (Nakamura and others, 2005). Similarly, using the minireplicon system for modelling influenza A and B viruses biological cycle, expression of canine Mx1 did not break the polymerase activity at all (Frensing and others, 2011; Seitz and others, 2010). In this study, the NP-positive cell fraction does not vary significantly in the presence of canine Mx1, suggesting, as in previous studies, that it is not endowed with anti-influenza activity.

In summary, all Mx1 proteins tested, with the notable exception of that of the dog, exert an anti-influenza activity, whatever the influenza virus they are exposed to, of avian or porcine origin. Since the experimental design is highly standardized and the expression levels of ectopic Mx1 were similar, both with respect to the transfected cell fraction and with respect to the average fluorescence

intensity emitted by the anti-V5 mAb, the amplitude of the measured anti-influenza effects can be compared. In doing so, it appears (i) that the anti-influenza effect is approximately similar for equine, bubaline and porcine Mx1 and (ii) that bovine Mx1 exerts an antiviral activity slightly greater than that shown by its three orthologs (Fig. 34). This last observation confirms previous studies (Garigliany and others, 2012; Palm and others, 2007). It should be noted that the readout used measures the rate of only a partial run of the viral biological cycle since the steps downstream of the nucleoprotein synthesis do not affect the results. Since it is known that at least one Mx1 protein plays a role in apical transport processes (Hoff and others, 2014), it is quite possible that certain Mx1 proteins may also brake assembly and/or budding processes.

To evaluate how the antiviral function and/or specificity of human MxA has been shaped during evolution of primates, an evolution-guided approach was implemented across 24 primate's MxA proteins to identify hotspots of recurrent positive selection. The study revealed that positive selection has acted on MxA at 12 discrete residues, likely in response to antagonistic evolution with viruses (Mitchell and others, 2012). The majority of positively selected sites are located in MxA stalk domain and, among them, by far the most striking enrichment of positively selected sites is in the unstructured loop L4, thus revealing a dramatic history of positive selection in L4 across primates. Further, grafting the L4 loop of an inactive MxA (that of *Chlorocebus pygerythrus*, african green monkey) or the single amino acid exchange F561V to human MxA decreased its restriction of influenza A virus replication. Conversely, adding the L4 region of (active) human MxA or only

A

Amino acid position #	18	35	196	301	401	409	416	540	561	564	566	567
Homo sapiens MxA	P	S	Y	I	I	H	T	G	F	F	S	S
Sus scrofa Mx1	H	T	Y	A	C	N	A	G	Y	S	P	A
Equus caballus Mx1	Q	R	R	A	T	Q	G	G	V	Q	T	S
Bos taurus Mx1	_	_	Y	I	S	A	A	R	Y	S	-	-
Bubalus bubalis Mx1	_	_	Y	I	S	T	A	R	Y	S	-	-
Canis lupus familiaris Mx1	H	_	R	D	S	N	A	R	M	D	V	S
Chlorocebus tantalus MxA	P	S	Y	I	S	R	I	G	V	F	P	S

B

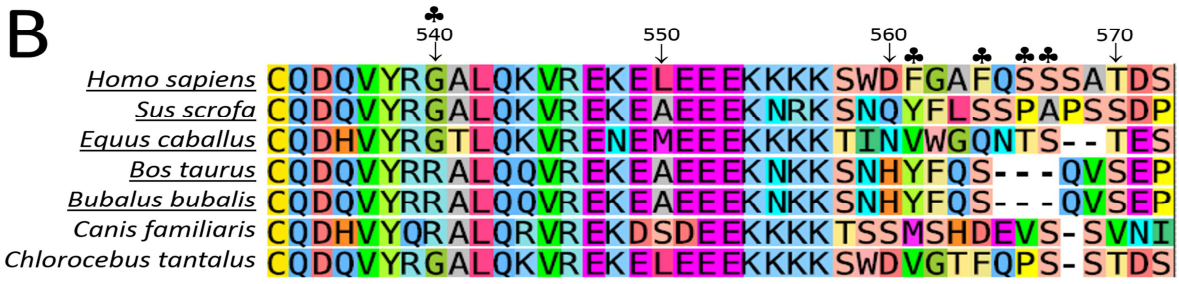


Figure 36: Inter-Mx1 comparison of the sites that evolved under strong potitive selection among primate MxA (Mitchell and others, 2012).

Notes: (A). The 12 antiviral residues spread over the entire human MxA. (B). The loop L4 (amino acids 533-572) protein sequence alignment. Residues and sequences are shown from influenza-inhibiting (human) or –noninhibiting (african green monkey) primate MxA and the set of influenza-inhibiting Mx1 orthologs studied here. Specific residues found to be evolving under positive selection in primate MxA are identified by the black clovers. Position numbering according to Mitchell and others (2012).

V561F to african green monkey MxA increased its antiviral activity to the same level as the wildtype human MxA protein (Mitchell and others, 2012). Taken together, these results are consistent with a generalized function for the loop L4, and for residue F561 in particular, in human MxA antiviral activity against and/or specificity for influenza A viruses.

Since influenza A viruses have always circulated between different species, especially between birds, pigs, horses, ruminants and humans, it is likely that the antagonistic arms race between Mx proteins and viral targets has simultaneously affected these different species. Since we have established that equine, bovine, bubaline and porcine Mx1 exert an anti-influenza function comparable to that associated with human MxA, we looked for common signature elements for these coevolutions. In doing so, we noticed that none of the 12 positively selected residues in primate MxA were strictly and systematically conserved (Fig. 35A), which at first sight suggests a lack of convergent coevolutions. Except that the F561 residue, critical for the anti-influenza function of primate MxA, becomes Y561 in anti-influenza bovine, bubaline and porcine Mx1 (Fig. 35B). Not in horses whose Mx1 displays anti-influenza activity as well, but we postulate that the adjacent aromatic residue, W562, absent from all other cytoplasmic Mx that has demonstrated anti-influenza activity, could functionally replace the aromatic residue (F or Y) present at position 561 in human, bovine, bubaline and porcine Mx1. In support of this hypothesis, the proven association between absence of anti-influenza function and absence of aromatic residues at positions 561-562 for MxA of african green monkey (Mitchell and others, 2012, Val-Gly) and the canine Mx1 (this study, Met-Ser). Therefore, by adopting an inter-species perspective, we suggest that it is the presence of an aromatic residue at positions 561 or 562 in the L4 loop that, in addition to the aforementioned requirements, is critical for the anti-influenza function and/or specificity of mammalian Mx1.

PERSPECTIVES

Despite of the time and financial constrain, with the huge afforce we have already obtained the valuably results including: two expression vectors of water buffalo Mx1 protein (bbMx1) in both prokaryotic and eukaryotic systems; bubaline mesenchymal stem cells, a set of 10 hybridomas secreting the 10 corresponding bbMx1-specific mAbs with the basic characteristics of each ligands. However, there are still some limitations, which need to be investigated continuously in the future studies to fulfil this type of research.

1. Check my final hypothesis that the presence of an aromatic residue at position 561 or 562 of any Mx1 protein is essential for the exercise of an anti-influenza activity

The L4 loop of Mx1 proteins is known to be essential for deploying an anti-influenza function. My dissertation led to the identification of two new mammalian Mx1s endowed with an anti-influenza function. So I was able to compare five distinct anti-influenza L4 loops with two L4 loops

deprived of anti-influenza activity. Hence the extraction of the common denominator from each other: to be active/inactive, an L4 loop must have an aromatic/nonaromatic residue in position 561 or 562. This conclusion obtained by successive deductions must now be confirmed by the experience. To achieve this, it would be necessary to synthesize an expression plasmid encoding a bovine, bubaline, equine, porcine or human Mx1 in which the aforementioned aromatic residue is replaced by one of the typical residues of inactive Mx1 proteins (canine or monkey). A "reverting" expression plasmid would also be synthesized, which would encode a canine Mx1 that would have the residue at position 561 or 562 “aromatized”. The two native proteins and the two mutated proteins would then be enlisted in one experiment in all similar to the one I conducted for my thesis.

2. Deepen the issue of the attachment platform

When an experimental confirmation will be obtained, the two molecular actors incriminated in the Mx1-dependent inhibition of the life cycle of the influenza A viruses will be known: the aromatic residue embedded in the L4 loop on the host side and the NP protein of the side of the virus. At this point, I think that fundamental studies could be undertaken by biochemists to discover the molecular mechanism of the interaction, for example using crystallography.

3. Defining the antiviral spectrum of bubaline Mx1

The antiviral activities displayed by buffalo Mx1 protein need to be investigated further with some other viruses, circulating dominantly in cattle and also supposed to do so in water buffalo. In this study, we have been able to conduct this type of experiment with influenza A viruses including H1N1 and H5N2 viruses. It will be much more valuable to continue the experiment with FMDV, corona virus, rotavirus, bovine herpes virus 1, (BHV-1) and bovine viral diarrhea virus, since these viruses have been found to circulate commonly in cattle in Asia. Therefore, future acquired results will have been not only fulfilling in our knowledge of the antiviral spectrum of the Mx1 protein but also might provide us more informations about mechanisms underlying the known intrinsic disease resistance of water buffaloes.

4. Defining the antiviral spectrum of bubaline Mx2

All mammals, with the notable exception of rodents, have two mx genes, which encode respectively the Mx1 and Mx2 proteins. Very few studies have been devoted to Mx2 proteins in general. There is certainly an extensive area of potential discoveries in this area.

5. Cooperation between ULiege and Hanoi University of Agriculture

I hope that the studies underlying my PhD dissertation, done under the auspices of the ARES, the University of Liège and the Agricultural University of Hanoi (Vietnam), will lead as quickly as possible to a bilateral cooperation aimed at transferring the prototype ELISA on the field in Vietnam. I also hope to develop new projects with the Faculty of Veterinary Medicine of University of Liège.

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