One Health 19 (2024) 100869

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

A multidisciplinary analysis of over 53,000 fascioliasis patients along the 1995–2019 countrywide spread in Vietnam defines a new epidemiological baseline for One Health approaches

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A R T I C L E I N F O

Keywords:
Human fascioliasis
Fasciola gigantica
Fasciola hybrids
Geographically spreading outbreak
Case number analyses
Spatio-temporal clustering
Incidence
Infection characteristics
Seasonality
Climate analyses and correlations
Risk indices
Disease transmission
Molecular characterization of fasciolids and lymnaeids
Radix viridis
Vegetable city markets
Livestock management and movements
Improving diagnostic capacity
Historical landmarks
Vietnam
South East Asia

A B S T R A C T

Fascioliasis, only foodborne trematodiasis of worldwide distribution, is caused by *Fasciola hepatica* and *F. gigantica*, liver flukes transmitted by freshwater snails. Southern and southeastern Asia is an emerging hot spot of *F. gigantica*, despite its hitherto less involvement in human infection. In Vietnam, increasing cases have been reported since 1995, whereas only sixteen throughout 1800–1994. A database was created to include epidemiological data of fascioliasis patients from the 63 Vietnam provinces throughout 1995–2019. Case profiles were based on serology, symptoms, eosinophilia, imaging techniques, stool egg finding, and post-specific-treatment recovery. Radio broadcasting about symptoms and costless diagnosis/treatment led patients to hospitals after symptom onset. Yearly case numbers were modelled and spatio-temporally analyzed. Missing data and confounders were assessed. The countrywide spread has no precedent. It started in the central coast, including 53,109 patients, mostly adults and females. Seasonality, linked to vegetable consumption, peaks in June, although the intensity of this peak differs according to relief/climatic zones. Incidence data and logistic regression curves are obtained for the first time in human fascioliasis. Fasciolid hybrids accompanying the spreading *F. gigantica* flukes, and climate change assessed by risk index correlations, are both ruled out as outbreak causes. Human-guided movements of livestock from an original area prove to be the way used by fasciolids and lymnaeid vectors to expand geographically. *Radix viridis*, a highly efficient transmitting and colonizing vector, played a decisive role in the spread. The use of irrigated crop fields, widely inhabited by *R. viridis*, for livestock grazing facilitated the transmission and spread of the disease. General physician awareness and diagnostic capacity improvement proved the successful impact of such knowledge transfer in facilitating and increasing patient infection detection. Information, education and communication to the public by radio broadcasting demonstrated to be very helpful. *Fasciola gigantica* is able to cause epidemic and endemic situations similar to *F. hepatica*. The magnitude of the human outbreak in Vietnam is a health wake-up call for southern and southeastern countries of Asia which present the highest human population densities with increasing food

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https://doi.org/10.1016/j.onehlt.2024.100869
Received 3 May 2024; Received in revised form 30 July 2024; Accepted 30 July 2024
Available online 3 August 2024

demands, uncontrolled livestock inter-country exchange, foreign import practices, and monsoon's increasing climate change impact.

1. Background

The liver flukes *Fasciola hepatica* and *F. gigantica* cause a freshwater-snail-borne zoonotic parasitic disease of great economic impact on livestock [1]. In humans, its high pathogenicity [2], sequelae [2,3], immune-suppression [4], impact on community development [5], increasing numbers of human endemic areas and case reports throughout 1990–2010, and worldwide distribution, unique among foodborne trematodiases [6], led WHO to include fascioliasis within neglected tropical diseases (NTDs) [7].

Fascioliasis is a highly complex disease with pronounced epidemiological heterogeneity and different transmission patterns. On one side, there are many biotic and abiotic factors playing a crucial role because it is a vector-borne disease transmitted by freshwater snails. These factors decisively influence:

– the phases of the life cycle of the fasciolid flukes,
– the lymnaeid snail vector populations and their dynamics, and
– the environmental habitats and climate characteristics of the transmission foci.

On another side, fascioliasis is a zoonotic disease and it is, moreover, a food- and water-borne disease [8]. There are, therefore, key aspects related to:

– the mammal animal reservoirs, among which mainly livestock such as sheep, goats, cattle, buffaloes, equines and Old-World camelids, but also omnivores as pigs [6], and
– the ethnographic characteristics of the inhabitants of the endemic rural area, including not only diet concerning vegetables, water and their combinations, but also behavioral traditions, social and religious aspects, attitudes, knowledge, livestock management practices, and even housing and home availability of electricity and running water [5].

All these numerous aspects differ according to endemic areas. Understanding the epidemiology and transmission of human fascioliasis in a given endemic area needs, therefore, multidisciplinary field surveys to be personally performed in the local environment, plus experimental studies to assess the peculiarities of the local fasciolids and their transmission by the local lymnaeids, all together within modern One Health assessments [9].

Fasciola gigantica is restricted to areas of Asia and Africa where it is transmitted by specific lymnaeid snail species of the *Radix* group [6]. It is more pathogenic than *F. hepatica*, although it has been always considered to be less involved in human infection [10]. Its higher development temperature thresholds underlie its historical spread as the only fasciolid across southern Asian warm lowlands [6,11], where increasing rainfall within climate change and livestock movements appear at present linked to an emerging fascioliasis hot spot eastward from Pakistan [12,13], and up to Vietnam. Climate changes markedly affect both the fasciolid life cycle and the population dynamics of its lymnaeid vectors, and lead to increasing prevalences, intensities and geographical spread of fascioliasis because of the absence of buffering premunition in the mammal hosts (mature egg-producing flukes accumulate in infected subjects because of the lack of coinfectious immunity, i.e. no buffer at the end of the life cycle) [9,11,14].

Among this wide Asian climate change vulnerable region, increasing human infection cases were reported in Vietnam since 1995 [15], whereas only sixteen cases, mostly Europeans, were reported throughout 1800–1994. Human infection in Vietnamese subjects was rarely reported since 1950, despite the common local infection in cattle.

The main aim of this study concerns the chronological follow-up of the geographical spread of human fascioliasis, since the beginning at the original focus of the outbreak in the year 1995 up to cover all the 63 provinces of the whole country in 2019. Quick reaction with radio broadcasting and early patient diagnosis and treatment in hospitals has allowed us to analyze a high number of cases. This primary end point includes the assessment of the epidemiological characteristics of a total of 53,109 cases and the analyses needed for the understanding of how a parasitic disease, which is not directly transmitted human to human but vector-borne, zoonotic and food/water-borne, is able to give rise to such

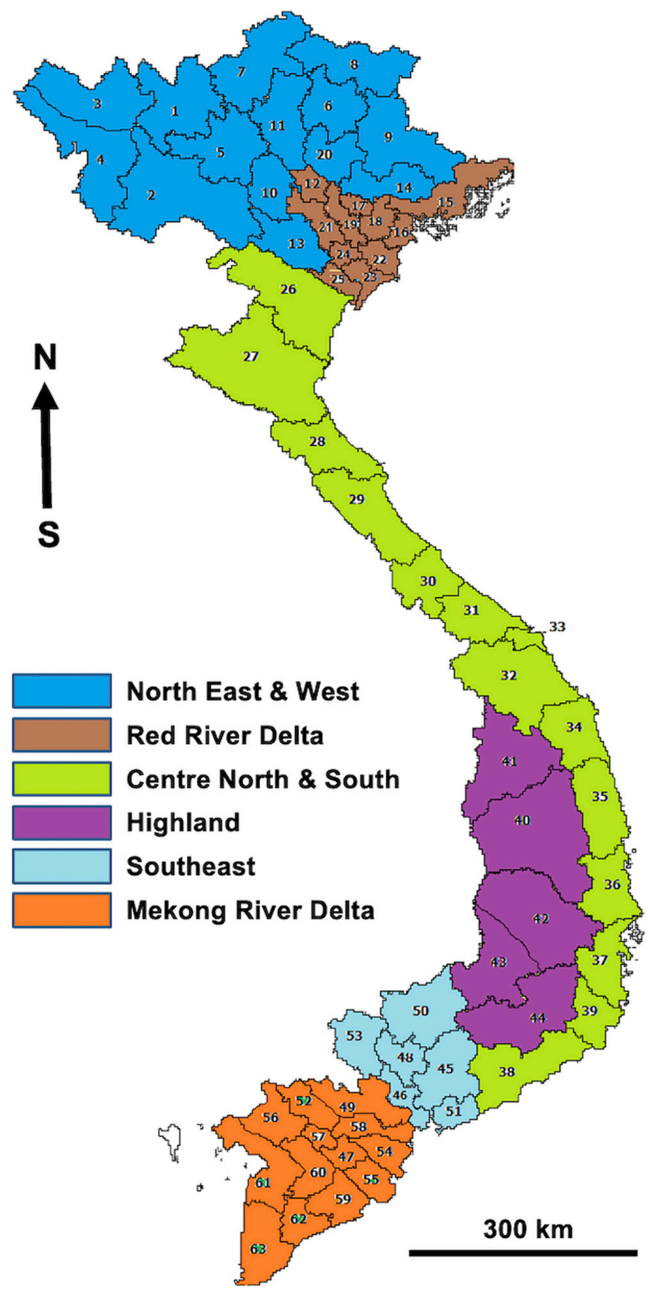

Fig. 1. Vietnam province map showing physiographic/climatic regions (black numbers correspond to provinces according to Table 1).

a geographically expanding epidemic in the context of a North-South outstretched country including different climate patterns as Vietnam (Fig. 1).

An additional interest relies on the geographical spread of genetically hybrid flukes after introduction of *F. hepatica* with imported livestock, a phenomenon accompanying the *F. gigantica* spread known to also occur in other southeastern Asian countries [6].

To understand the action steps undertaken for the follow-up of the countrywide outbreak of human fascioliasis in Vietnam and the progressive design of control measures to face it, several historical landmarks and the chronological implementation of activities within the country reaction should be duly analyzed and considered.

The importance of several results obtained in the analysis of the human fascioliasis outbreak in Vietnam transcend the country and even the regional scope. Many new aspects should be incorporated to the epidemiological baseline of this disease and also henceforth considered within the modern One Health multidisciplinary approaches highlighted to be needed for the prevention and control of this highly complex disease [9].

2. Material and methods

2.1. Analytical procedures

Records of *Fasciola*-infected patients were registered in hospital cards in the 63 Vietnam provinces. A national database was created for the registry of epidemiological and clinical data of 53,109 patients diagnosed throughout 1995–2019. Missing data concerning possibly overlooked initial individual infections in each province were considered (Supplement 1), and all causes and confounders potentially underlying curve abnormalities were assessed (Supplement 1). Years from 2020 onwards are excluded to avoid bias due to a potential impact of the COVID-19 pandemic.

2.2. Patients

2.2.1. Eligibility criteria and sample size

Case profile was based on symptoms, eosinophilia, serology, imaging techniques, stool egg finding, and post-specific-treatment recovery. Broadcasting about symptoms and costless diagnosis and treatment led patients to go to hospitals after symptom onset. Egaten® (triclabendazole for human use, Novartis Pharma, Basel) was provided free of charge by WHO to hospitals. The eligibility criteria for the selection of the *Fasciola*-infected cases were of well-known usefulness for the diagnosis of human fascioliasis [16] and included:

– the typical symptoms in the acute and chronic phases of the liver fluke infection [2,17,18];

- eosinophilia as a very useful marker due to its early appearance in fascioliasis [2,17];
- positivity in a serological test which was systematically applied to suspected patients; the ELISA test based on a Vietnamese *F. gigantica* excretory/secretory protein showed 100% sensitivity and 97.67% specificity [19];
- ultrasonography available in almost all provincial hospitals, and computed tomography and magnetic resonance in well-equipped city hospitals, furnished hepatic abnormality findings suggestive of fascioliasis [17,18,20];
- the coprological Kato-Katz technique was used for the search of fasciolid eggs [16];
- disappearance of symptoms after treatment with the anti-fasciolid specific triclabendazole, highly efficient in all disease phases [21], provided infection ex-juvantibus confirmation [2].

Individual patient information on epidemiological and clinical data recorded in specific cards from hospitals of all the 63 provinces of Vietnam were collected by the author's centers in Ha Noi, Quy Nhon and Ho Chi Minh and allowed for the construction of the appropriate countrywide database. The quick reaction of Vietnam after the detection of initial cases in the central coastal provinces of Da Nang and Binh Dinh since 1995 allowed for the follow-up of the outbreak since its beginning.

The sample size of 53,109 cases for the outbreak analysis is defined by the sum of cases diagnosed in the total of 63 provinces of Vietnam and the period elapsed between the outbreak beginning (i.e. first cases diagnosed) in 1995 and 2019. The potential impact of the COVID-19 pandemic suggested that it was better to exclude from the year 2020 onwards to avoid biases.

2.2.2. Missing data

Regarding missing data, see Supplement 1 and consider related references [2, 17, 18, 22, 23, 24, 25, 26, 27, 28].

2.2.3. Case number analyses and confounding assessments

Potential confounding factors are detailed in Supplement 1.

2.2.4. Case number evolutionary scenarios according to zones

To assess the epidemiological pattern followed by the annual number of cases per province, logistic regression curves were obtained by means of IBM SPSS Statistics version 28.0.1.1. In these analyses, statistical significance was evaluated by considering a P value of <0.05.

Four zones of Vietnam were analyzed by considering the corresponding selected groups of provinces. The provinces selected were only those in which infected patients began to be detected before 2006 and the annual number of cases proved to have reached a final plateau. This plateau refers to the several subsequent years along which the annual case number appears to be stabilized, with only slight increases/decreases per year. In zones in which more than one province was included, a mean logistic curve was obtained from the mean annual values of all provinces considered.

2.2.5. Disease incidence analyses

Human fascioliasis has been a neglected disease for decades. The discovery of human fascioliasis endemic areas only very recently, and the fact of being a non-reportable disease, explain why studies assessing incidence are lacking.

The present study of patients diagnosed yearly in Vietnam provinces along the 1995–2019 period offers therefore the first occasion to analyze real incidence data from new cases per year per province in different physiographic/climatic zones of a low-income country. For this analysis, provinces still showing a strongly increasing yearly case number in 2019 (still in the epidemic period), namely in 16 among the total of 63 provinces, were not considered. The remaining 47 provinces in which the yearly case number reached an evident, more or less stabilized, final plateau of several years (endemic period) were computed.

The incidence was estimated from the mean of new cases/year/province along the years in which the province may be considered in the aforementioned endemic period. For the final calculation, the number of inhabitants of each province in each of the aforementioned years was obtained from the website of the Vietnam General Statistics Office (https://www.gso.gov.vn/en/homepage/). Incidence data were finally referred to 100,000 people-year.

2.3. Analyses of infection characteristics

Infection characteristics were studied regarding sex, age, seasonality and provinces, crucial features linked to inhabitant ethnography and in need to be considered within One Health assessments [5,9].

In human fascioliasis, infection difference according to sex is a core epidemiological characteristic with impact on the gender role in rural communities. Higher infection rates in females are well known in rural communities suffering human hyperendemicity for a long time. In the Northern Altiplano of Bolivia, markedly higher intensities were reported in females [24], whereas in the Nile Delta, Egypt, it was the prevalences that were significantly higher in females [27]. Reports referring to higher prevalences in females in other countries are numerous [5,24,27]. Long ethnographic studies in the rural communities have demonstrated that the higher infection risk in girls and women is related to their traditional roles in food preparation and work in the kitchen [5]. Gender was, therefore, considered as the crucial variable to be compared with the other factors.

Infection rates according to age vary in the different endemic areas. In given areas, children are the main group affected by this disease, as in Bolivia [24], Peru [25,29], or Egypt [27], whereas in other areas it is for adult subjects to be the most infected group, as in Iran [30] or Pakistan [12].

Seasonality is another important epidemiological factor to be analyzed in fascioliasis. Seasonal temperatures and rainfall (i.e., surface water availability) define both the lymnaeid snail vector population dynamics and the growing periods of fasciolid metacercariae-carrier vegetables, and consequently the annual seasons of human and animal infection. The seasonality was analyzed by considering the hospital admission month of each patient.

Statistical analyses were made using GraphPad Prism version 9.5.1 for MacOS (GraphPad Software, San Diego, California USA, www.graphpad.com).

The Chi-square test and Fisher's exact test were used for the evaluation of categorical variables, as long as the available sample size meets the requirements needed for the test to be applied. The number of fascioliasis patients (N), fascioliasis proportion (%), confidence interval (CI) (% 95) and comparison of proportions were analyzed. A P value of <0.05 was considered significant in these statistical analyses (two-sided when applying Fisher's exact test).

2.4. Spatio-temporal clustering

To analyze the temporal and spatial distribution of cases, spatio-temporal statistics were employed. Spatio-temporal statistics allow the analysis of basic rate maps of disease to determine whether the observed space-time patterns of a disease are randomly distributed or not. The detection of spatio-temporal clusters identifies areas or time periods with abnormally high incidence in order to understand the underlying causes of the disease.

A retrospective space–time scan statistic (STSS), based on a Poisson model, was performed to identify the presence of high-risk space-time clusters of human fascioliasis cases in Vietnam during the period 1995–2019. A Poisson-based model assumes that the number of events in a geographical area is Poisson-distributed, according to a known underlying population at risk [31]. The yearly population data for the period 1995–2019, detailed by province, was obtained from the website of the Vietnam General Statistics Office (https://www.gso.gov.vn/en/homepage/).

Space–time scanning, defined as a dynamic scan using a cylindrical window in dimensions of time scales and geographical locations, was used for the identification of spatio-temporal disease clusters [31,32]. For each scanning window, a log likelihood ratio (LLR) was calculated to test for elevated risk within the window in comparison to outside the window [31,32]. A P value <0.05 was indicative of a statistically significant cluster. According to the P value and LLR value, it is possible to determine whether there is clustering in the study area, and, moreover, to analyze its exact location and risk size. Cluster analyses were performed by means of SaTScan software version 10.1 (http://www.satscan.org/).

2.5. Climate analyses and correlations

Both transmission and epidemiology of fascioliasis are pronouncedly dependent on climatic factors [33]. Therefore, when dealing on this disease, assessing a potential correlation between climate changes and the emergence of fascioliasis is necessary. Moreover, countries of southern and south-eastern Asia are climatologically characterized by the monsoon, with very pronounced increases of rainfall rates in recent years. This facilitates lymnaeid snail vector population increases, in its turn increasing liver fluke transmission rates and leading to higher infection risk [11,13,34].

Therefore, we have performed deep analyses of the climate change experienced by the different climate zones of Vietnam during the last decades and assessed whether they could be the cause of the wide human outbreak and spread throughout this climatologically heterogeneous country.

Climatic factor data concerning the period 1994–2019 were obtained from meteorological stations located in Vietnam and neighboring countries (Fig. 2, Supplement 3). Two fascioliasis risk indices were used for the correlation analyses of the evolutionary curve of fascioliasis patients per province: the Wet Day (Mt) forecast index and the Water-Budget-Based System (Wb-bs) forecast index (Supplement 3). Respective calculation methods are noted in Supplement 3 and related references [11, 35, 36, 37, 38, 39, 40, 41]. The monthly fascioliasis risk indices obtained from meteorological stations data were summarized in several annual measurements to be later used in the analysis of correlation with the evolutionary curve of yearly fascioliasis cases per province (Supplement 3). The yearly curve of fascioliasis patients per province was correlated with respective yearly measurements of risk indices by means of linear regressions, by considering time-to-event periods according to the *Fasciola* life cycle length (Supplement 3).

2.6. Field studies to assess the disease transmission

We performed field surveys in rural and peri-urban areas of the provinces of Binh Dinh, Da Nang and Ha Noi, including sampling of fasciolid flukes from slaughtered livestock and lymnaeid snails collected

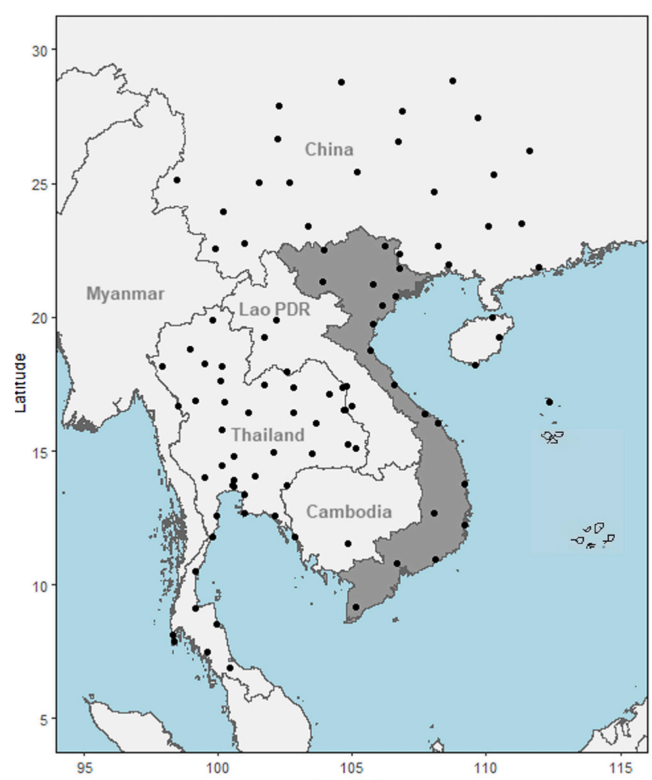

Fig. 2. Map of Vietnam (filled in dark grey) and neighboring countries, showing the location of the meteorological stations whose climatic data were used for the calculation of fascioliasis risk indices.

in field studies, plus surveys of crop fields under intense irrigation to look for lymnaeid populations and their local environment, vegetable species involved, and livestock management practices linked to fascioliasis transmission and human infection sources. These field analyses were performed by experienced personnel of the Valencia WHO Collaborating Centre focusing on aspects known to be crucial for disease transmission and human infection sources, as already reported to have been followed in One Health assessments in other human fascioliasis endemic areas [5].

2.6.1. DNA marker sequencing of animal fasciolids and lymnaeid snails

In Asia, the presence of *F. hepatica*, *F. gigantica* and hybrid *Fasciola* flukes leads to the need of DNA marker sequencing for the classification of fasciolid specimens. The nuclear ribosomal DNA internal transcribed spacers ITS-2 and ITS-1 and the mitochondrial DNA coding genes *cox*1 and *nad*1 are the markers used for this purpose [42,43]. These four markers were sequenced from fasciolid specimens found in the livers of seven local cattle from Binh Dinh, one cow from Quang Nam, and two cows from Ha Noi, all collected during the 2007 WHO mission.

The ribosomal DNA ITS-2 is the marker of choice for lymnaeid species classification [44] and was used for the lymnaeids collected in the fascioliasis transmission foci in the aforementioned three provinces of Vietnam. Overall, 20 specimens from each water collection around the localities of Hai Boi, Dong Anh, Ha Noi and Tuy Phuoc, Quy Nhon, Binh Dinh, were randomly selected to be representative samples covering the north and central Vietnam, respectively.

DNA was extracted individually from each fluke and snail with the phenol-chloroform method. DNA was precipitated with ethanol and was PCR amplified independently for each specimen using primers previously described [43,45]. Amplification procedures and thermal cycler conditions were carried out in a Mastercycle epgradient (Eppendorf, Hamburg, Germany) [46,47]. PCR products were purified using the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) and resuspended in 50 μl of 10 mM TE buffer (pH 7.6). The final DNA concentration (in μg/ml) and the absorbance at 260/280 nm were determined in an Eppendorf BioPhotometer (Hamburg, Germany). Sequencing was performed on both strands by the dideoxy chain-termination method with the Taq dye-terminator chemistry kit on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using PCR primers.

The software Sequencher v. 5.4.6 (Gene Codes Co. MI, USA) was used to edit and assemble the sequences and ClustalW to align them by means of default parameters in MEGA X. Homologies were assessed using the BLASTN program from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

2.6.2. Lymnaeid vector ecology

The ecological preferences of Vietnamese *R. viridis* populations were assessed by surveying the rural and peri-urban zones of Binh Dinh, Da Nang and Ha Noi. Field studies were performed during sunshine hours and focused on environmental places presenting characteristics of fascioliasis transmission foci, including superficial freshwater collections frequented by livestock and humans [48]. Localities were georeferenced and respective photographical records archived.

2.6.3. Vegetable production and consumption

Surveyed crop fields inhabited by lymnaeid populations were assessed regarding species of vegetables grown, irrigation systems, presence of farm workers and livestock, and their geographical distribution throughout rural and peri-urban zones of Binh Dinh, Da Nang and Ha Noi. A special focus was devoted to city markets where vegetables were sold without any previous health control.

2.6.4. Livestock management

Human management and human-guided movements of domestic animal reservoirs are known to be key aspects in (i) keeping fascioliasis endemicity [5,49,50], and (ii) spreading the geographical distribution of the disease [6,51], respectively. We analyzed the human management of domestic ruminants concerning (i) livestock infection sources, (ii) animal contribution to disease transmission, and (iii) large ruminant participation in the spread of fascioliasis, both at local level and trans-province countrywide level. Corresponding photographical records were obtained and archived.

3. Results

3.1. Analysis of the starting situation

Crucial historical landmarks and past reports which should be considered to understand the situation of Vietnam before the human outbreak are detailed in Supplement 2 and related references [6, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61].

3.1.1. Beginning of the human fascioliasis outbreak (1995–2003)

The human fascioliasis situation in Vietnam began to change at the end of the 20th century, after the finding of two *F. gigantica* adult specimens in the liver of a patient surgically-intervened at Da Nang hospital in 1995 (De et al., unpublished). From 1996 to 2000, the number of patients diagnosed to be infected by fasciolids began to increase in the central and south-central provinces.

Around 500 cases of human fascioliasis were initially reported from hospitals in 2001 [62]. Patients infected in Vietnam also began to be diagnosed abroad [63]. A first community survey in 2002 showed a worrying human fascioliasis prevalence of 11% in Khanh Hoa province [64].

The fasciolid species involved in the human and cattle infections was preliminarily clarified in articles locally published in Vietnamese in 2001 in which the sequencing of a fragment of the mtDNA *cox*1 gene demonstrated that it was *F. gigantica* in all cases [60].

3.1.2. Reasons why the country was not ready

The following crucial aspects from the historical frame of fascioliasis during the 19th and 20th centuries should be considered to understand why Vietnam was not ready to face fascioliasis at the beginning of the outbreak and was needed to launch a strong outstanding country reaction:

– Human fascioliasis had caused only sporadic infections and was therefore never considered a public health problem in Vietnam before.

– Liver trematodiasis in this country was generally considered to be caused by *Clonorchis sinensis* and sporadically *Opisthorchis viverrini* [64], both well-known among physicians because of causing cholangiocarcinoma [65,66].

– Vietnamese physicians were not aware about the existence in the country of a liver fluke able to infect humans and belonging to another trematode species.

– Hospitals were not ready to face a fascioliasis outbreak, neither concerning a differential clinical diagnosis nor on how to parasitologically and/or serologically diagnose this disease.

– The lack of updated specific literature in the Vietnamese language was an important obstacle to physicians and public health personnel accessing the appropriate information.

– Similarly, rural and urban inhabitants were not aware of fascioliasis and its transmission and consequently nobody dedicated attention to the risk associated with the consumption of intense-irrigation-needing vegetables, freshwater vegetables, or natural water drinking [8].

3.2. The countrywide spread

A total of 53,109 patients were diagnosed with fasciolids in the 63 provinces throughout 1995–2019 (Figs. 3 and 4). Central-south coastal provinces show the highest accumulated number of cases. From the first two 1995 cases in Da Nang Nang, it gradually expanded to cover initially

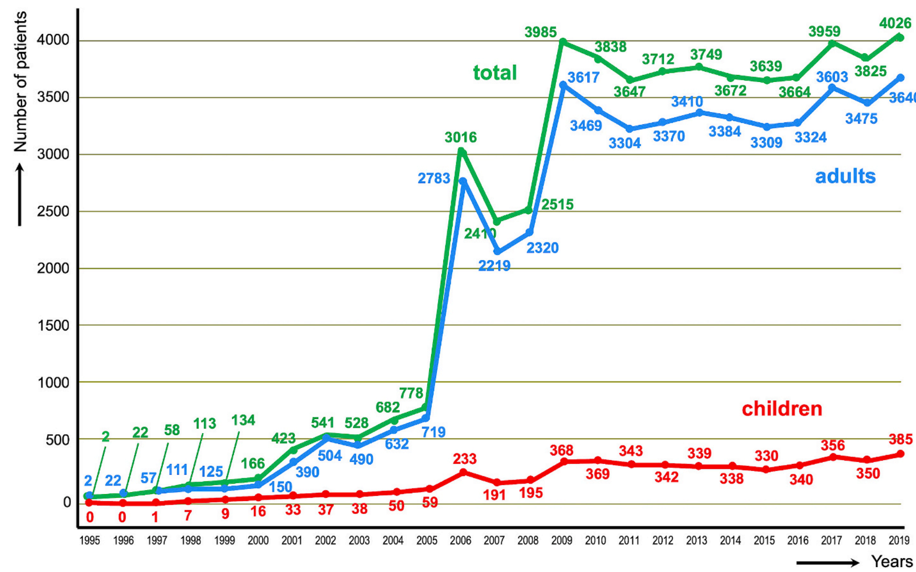

Fig. 3. Evolution of the yearly number of cases throughout the country of Vietnam along 1995–2019 in total patients (green curve), adult subjects (blue curve) and children (red curve).

all southern warmer provinces and subsequently the whole northern cooler Vietnam (Table 1).

Case number, whether total, in adults or children, shows marked increases in 2006 and 2009 (Fig. 3). In many provinces, patients began to be diagnosed only from 2006 (Table 1). The time needed from the first case (epidemic period) up to reach a yearly case number plateau (endemicity period) in a province ranged 2–12 years (mean 6.4 years), although, in several provinces, cases were still increasing in 2019 (Table 1).

3.2.1. Discarding confounding factors in marked yearly case number rebounds

Assessments made to discard such confounding factors are noted in Supplement 1.

3.2.2. Increased awareness and diagnostic training as rebound causes

The only remaining potential cause of the 2006 and 2009 rebounds is related to an increase of patient detection thanks to improvements in the physician awareness and diagnostic capacity.

Chronological evidence indicates the 2006 rebound to have been the consequence of efforts in the distribution of 400 copies of a monographic book on liver trematodiases written by the first and third authors and published in Vietnamese in 2004 to all provinces, including hospitals and physicians, especially clinicians. Health professionals attribute the improved awareness of fascioliasis emergence attained among hospital physicians to the countrywide diffusion of this book. This book, together with clinical parasitology teaching courses by the first author (NVD) in several hospitals of the capital Ha Noi and provinces, led local medical professionals to consider fascioliasis for the first time within liver fluke diseases in Vietnam.

A retrospective analysis of the situation in Vietnam during the years previous to 2009 additionally indicates an impact by the March–April 2007 WHO specific mission. This mission by experts of the Valencia WHO Collaborating Centre on fascioliasis plus WHO Headquarters Geneva and WHO-WPRO Ha Noi staff members included monographic meetings in Ha Noi with national health authorities of Vietnam, additional meetings and conferences to the staff physician personnel of the Institute of Malariology, Parasitology and Entomology and hospitals of Quy Nhon and Da Nang, plus laboratory training about human fascioliasis diagnostic methods to the technical staffs of the aforementioned centers of Ha Noi, Quy Nhon and Da Nang.

3.2.3. Case number curve modelling

The four logistic regression curves obtained according to the selected zones (Fig. 5A), including only provinces in which infected patients began to be detected before 2006 and the annual number of cases proved to have reached a final plateau, proved to be significant (Table 2).

When investigating changes of the number of fascioliasis cases along time, all the resulting curves proved to be logistic, according to a model following an initial slowly increasing dissemination phase, a subsequent faster increasing epidemic phase, and a final stabilized plateau endemic phase. Curve slopes decrease as we move away from the central coast where the first cases were detected. The value of y_m of the logistic regression curve is highest in the Central Coast of Vietnam (province No. 35 in Table 2), lower in Central South and Highlands (provinces 34, 36, 37, 42, 44), and even lower in provinces of the Central North and North (provinces 18, 24, 27, 28, 30), and South (provinces 45, 46, 51, 53, 54).

3.2.4. Incidence per provinces

The incidence estimate (mean number of new cases/province/year along the endemicity period) ranges 0.55–24.95/100,000 person-year according to the 47 provinces in which this assessment could be performed (Table 2), with a countrywide mean of 6.85/100,000 inhabitants-year.

The incidence per province proved to be lower than 1.00/100,000 person-year only in the provinces of the two big cities of Ha Noi (0.93/100,000 person-year) and Ho Chi Minh (0.55/100,000 person-year), higher than 10.00/100,000 person-year in the coastal provinces, and with the highest incidences close to or higher than 20.00/100,000 person-year in the coastal provinces of Binh Dinh (23.71/100,000 person-year) and Phu Yen (19.76/100,000 person-year), and the highland provinces of Kon Tum (24.95/100,000 person-year) and Dak Nong (19.60/100,000 person-year). These later four provinces are indeed among those in which human fascioliasis spread was earlier detected.

It should be highlighted that the yearly case numbers along the endemic period inside each of the 47 provinces only show reduced fluctuations.

3.2.5. Cluster analyses

Space-time scan analysis identified twelve significant clusters of human fascioliasis cases between 1995 and 2019 (Table 3, Fig. 5B), varying in their time-period length and radius. These clustering results indicate that human fascioliasis cases within each significant spatio-

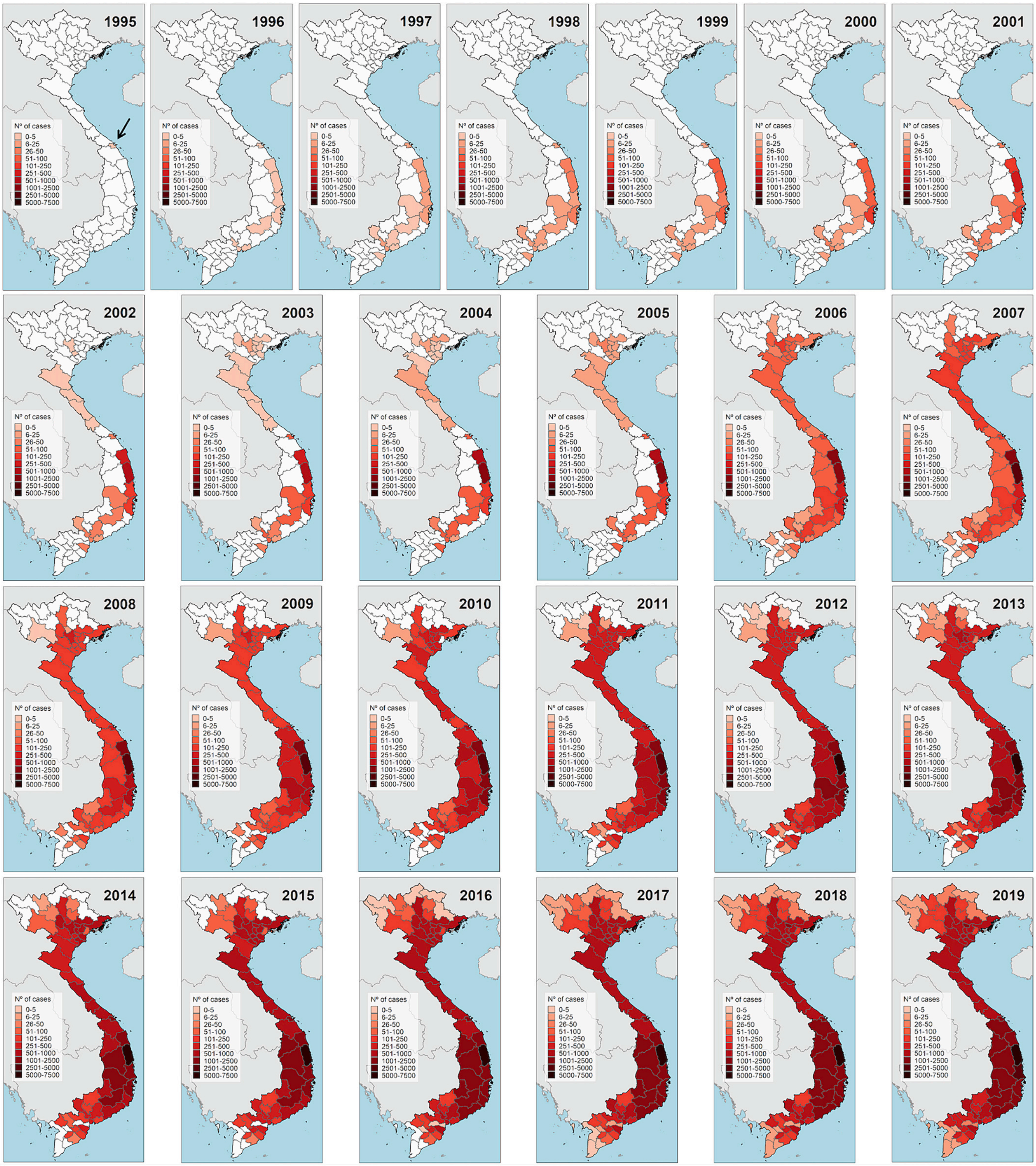

Fig. 4. Spread of human fascioliasis throughout the provinces of Vietnam along the 1995–2019 period. From the first two 1995 cases in Da Nang Nang (black arrow), it gradually expanded to cover all southern warmer provinces and whole northern cooler Vietnam.

temporal cluster are grouped together in space and time, with an underlying cause different than chance.

The first significant cluster was detected between 2001 and 2012 and involved the provinces of Binh Dinh, Gia Lai, and Quang Ngai. The persons present in the area during that time-period were 9.85 times more likely of acquiring fascioliasis. It is noteworthy that ten of the following significant clusters started in 2006, being scattered through the country and showing a similar duration.

After discarding all potential confounding factors (see Supplement 1), the wide distribution of the aforementioned 2004 monographic book on liver trematodiases and the impact of the March–April 2007 WHO mission are the only remaining factors which appear associated with the increased detection of human fascioliasis cases evidenced by these spatio-temporal clusters, both with a time-to-event effect of two years.

Table 1
Human fascioliasis cases in each province of Vietnam along the 1995–2019 period.

A	B	C	D	E	F	G	H	I
1	Lao Cai	2012	28	151	s.i.	–	–	
2	Son La	2008	31	200	9	29.7	2.41	
3	Lai Chau	2016	6	18	s.i.	–	–	
4	Dien Bien	2016	8	22	s.i.	–	–	
5	Yen Bai	2011	22	118	4	17.6	2.20	
6	Bac Kan	2012	25	137	2	19.0	6.02	
7	Ha Giang	2016	10	28	s.i.	–	–	
8	Cao Bang	2016	9	21	s.i.	–	–	
9	Lang Son	2016	21	50	s.i.	–	–	
10	Phu Tho	2003	83	885	7	70.4	5.15	
11	Tuyen Quang	2005	68	712	4	58.3	7.72	
12	Vinh Phuc	2008	82	877	3	78.9	7.47	
13	Hoa Binh	2006	74	813	4	64.6	7.89	
14	Bac Giang	2003	73	878	4	61.5	3.80	
15	Quang Ninh	2006	98	929	6	79.0	6.46	
16	Hai Phong	2011	31	209	s.i.	–	–	
17	Bac Ninh	2002	112	1141	11	100.0	8.42	0.63
18	Hai Duong	2001	70	800	6	56.2	3.20	0.37
19	Hung Yen	2003	86	985	4	69.6	6.02	
20	Thai Nguyen	2011	30	217	3	26.3	2.14	
21	Ha Noi	2002	82	933	5	64.3	0.93	
22	Thai Binh	2002	75	880	5	62.2	3.47	
23	Nam Dinh	2003	73	873	4	61.6	3.36	0.59
24	Ha Nam	2001	70	805	6	57.0	7.13	
25	Ninh Binh	2006	63	731	0	52.2	5.62	
26	Thanh Hoa	2002	70	658	5	46.0	1.32	0.51
27	Nghe An	2001	74	835	6	58.8	1.94	0.42
28	Ha Tinh	2002	75	826	6	58.3	4.65	
29	Quang Binh	2002	76	868	5	61.3	7.12	0.52
30	Quang Tri	2001	88	967	6	68.4	11.19	
31	Thua Thien Hue	2006	91	921	0	65.8	5.89	
32	Quang Nam	2006	108	1097	0	78.3	5.38	
33	Da Nang	1995	143	1760	12	120.8	12.42	0.45
34	Quang Ngai	1996	204	2472	6	126.2	10.30	
35	Binh Dinh	1996	748	6822	6	354.6	23.71	0.45
36	Phu Yen	1996	231	2673	11	173.3	19.76	
37	Khanh Hoa	1996	288	2339	11	149.9	12.66	0.36
38	Binh Thuan	2006	125	1349	0	96.3	8.07	
39	Ninh Thuan	2006	150	1375	0	98.2	16.89	
40	Gia Lai	2006	172	1659	0	118.5	8.78	
41	Kon Tum	2006	192	1640	0	117.1	24.95	
42	Dak Lak	1996	251	1997	11	136.6	7.59	0.53
43	Dak Nong	2006	150	1498	0	107.0	19.60	
44	Lam Dong	1996	222	1936	11	132.3	10.71	
45	Dong Nai	1996	75	734	11	46.7	1.71	
46	Ho Chi Minh	1996	60	687	11	42.3	0.55	0.41
47	Vinh Long	2006	33	372	0	26.5	2.57	
48	Binh Duong	2006	30	330	0	23.6	1.34	
49	Long An	2006	36	371	0	26.5	1.80	
50	Binh Phuoc	2006	28	271	0	19.6	2.12	
51	Ba Ria-Vung Tau	1996	55	445	s.i.	–	–	
52	Dong Thap	2011	48	280	s.i.	–	–	
53	Tay Ninh	1996	59	657	11	44.6	4.07	
54	Ben Tre	1996	78	788	s.i.	–	–	
55	Tra Vinh	2006	27	271	0	19.3	1.90	
56	An Giang	2006	29	293	2	13.0	1.03	
57	Hau Giang	2006	30	283	2	21.2	2.77	
58	Tien Giang	2016	13	27	s.i.	–	–	
59	Soc Trang	2011	28	141	s.i.	–	–	
60	Can Tho	2016	11	25	s.i.	–	–	
61	Kien Giang	2017	5	12	s.i.	–	–	
62	Bac Lieu	2016	7	13	s.i.	–	–	
63	Ca Mau	2016	6	14	s.i.	–	–	

A: number in map of Fig. 1; B: province; C: first year in which a human case was diagnosed; D: maximum number of cases in one year; E: total number of accumulated cases until 2019; F: years needed until reaching a stabilized plateau (epidemic phase) (provinces showing 0 share 2006 in C, which suggests cases overlooked before); G: mean number of new cases/year in the stabilized plateau (endemic phase); H: Incidence in cases/100,000 inhabitants-year during the endemic phase (population per province in thousands of persons in 2019 obtained from General Statistics Office of Vietnam - https://www.gso.gov.vn/en/data-and-statistics/2020/09/23373/); I: significant correlation value between monthly risk indices and monthly human cases throughout the endemic phase in the only eleven provinces including a meteorological station and showing an initial gradual increasing yearly case number (see time-to-events). s.i. = yearly case number still increasing in 2019; –- = stabilized plateau not reached.

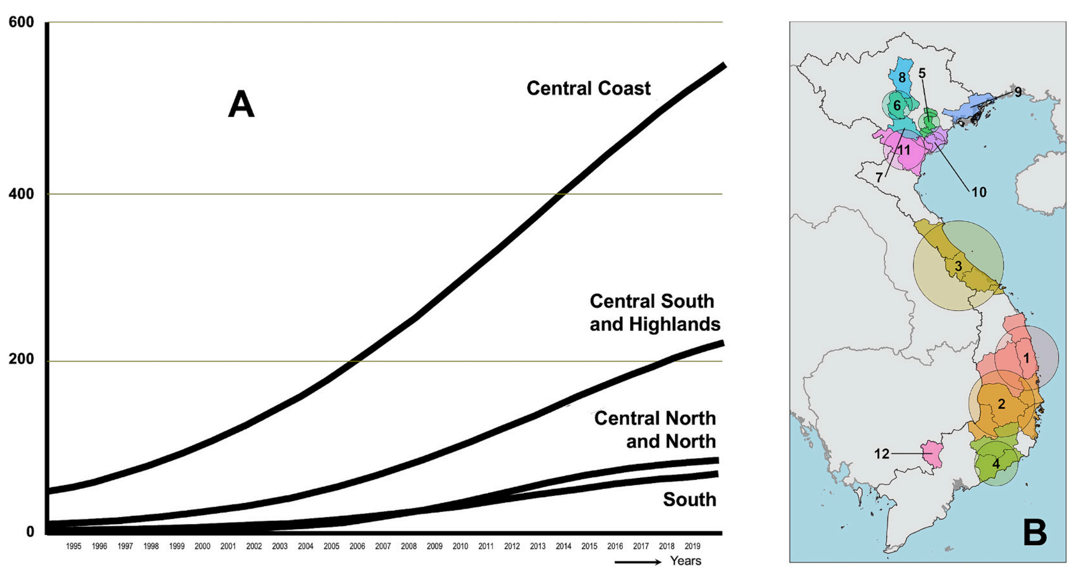

Fig. 5. Modelling human fascioliasis in Vietnam: A) logistic epidemiological curve variations according to country zones (including only provinces in which infected patients began to be detected before 2006 and the annual number of cases proved to have reached a final plateau (see Table 1); B) Geographical representation of the twelve significant spatio-temporal clusters of human fascioliasis cases found between 1995 and 2019 (circles represent the cluster radius in km when >0; same color provinces pertain to the same cluster).

Table 2
Comparison of the logistic regression curves and their significance values according to zones of Vietnam including only provinces in which infected patients began to be detected before 2006 and the annual number of cases proved to have reached a final plateau.

Zones of Vietnam	Provinces considered	y_m	z_0	k	R	ANOVA significance	Coefficient significance
Central Coast	35 – Binh Dinh	750	0.869	0.656	0.422	0.040	<0.001
Central South and Highlands	34 – Quang Ngai 36 – Phu Yen 37 – Khanh Hoa 42 – Dak Lak 44 – Lam Dong	290	0.835	0.425	0.855	<0.001	<0.001
South	45 – Dong Nai 46 – TP. Ho Chi Minh 51 – Vinh Long 53 – Tay Ninh 54 – Ben Tre	80	0.804	0.376	0.979	<0.001	<0.001
Central North and North	18 – Hai Duong 24 – Ha Nam 27 – Nghe An 28 – Ha Tinh 30 – Quang Tri	90	0.730	0.433	0.838	<0.001	<0.001

$Y = y_m/[1 + z_0 \exp.(-kt)]$, where: ym = the maximum value attainable by the variable y (= number of people affected); t = years.

Table 3
Details of the spatio-temporal clustering analysis of human fascioliasis cases in Vietnam from 1995 to 2019.

#	Period	Cluster center		Radius (km)	Clustered provinces	No. of cases	No. of expected cases	Relative risk	P value
		Latitude	Longitude						
1	2001–2012	14.122	108.951	102	Binh Dinh, Gia Lai, Quang Ngai	7595	884.9	9.85	<0.001
2	2006–2017	12.825	108.207	105.7	Dak Lak, Dak Nong, Phu Yen, Khanh Hoa	6587	1653.1	4.41	<0.001
3	2006–2017	16.746	106.930	142.2	Quang Tri, Thua Thien Hue, Quang Binh, Da Nang	3689	1334	2.90	<0.001
4	2006–2017	11.118	108.048	70.6	Binh Thuan, Lam Dong	2766	910.5	3.15	<0.001
5	2006–2016	20.815	106.060	33.1	Hung Yen, Ha Nam, Bac Ninh	2182	978	2.28	<0.001
6	2009–2015	21.319	105.117	46.1	Phu Tho, Vinh Phuc	1023	522.4	1.98	<0.001
7	2006–2017	20.684	105.344	0	Hoa Binh	673	305.8	2.22	<0.001
8	2006–2015	22.113	105.268	0	Tuyen Quang	494	203.9	2.44	<0.001
9	2006–2015	21.247	107.284	0	Quang Ninh	638	322.9	1.99	<0.001
10	2006–2012	20.268	106.216	32.2	Nam Dinh, Thai Binh	816	586.8	1.40	<0.001
11	2006	20.046	105.320	63.9	Thanh Hoa, Ninh Binh	133	72.2	1.84	<0.001
12	2006–2013	11.404	106.162	0	Tay Ninh	309	211.1	1.47	<0.001

All the clusters detected, especially the first one, coincide with the areas more heavily affected by the disease.

3.2.6. Infection according to sex and age

Infection rates are higher in females and in adult subjects, including from children aged <1 year up to an 88-year-old patient (Fig. 6A). Within each age group, female patients are always markedly more numerous than male patients. The differences between their respective proportions prove to be significant in all age groups (Table 4, Fig. 7A). This means that the behavior of females and males linked to infection sources do not change according to age in Vietnam. This suggests a similar consumption of vegetables by all members of the familiar unit.

Two aspects should be highlighted in Vietnam: (i) the proportion of infected women is markedly higher than the proportion of men in all age groups (with a mean proportion in females around the double than males in all age groups - 66% versus 33%) (Table 4, Fig. 7A), and (ii) no significant differences are detected in the comparison between the age groups (Chi square test, $P = 0.309$), although the values of the prevalence curve show their maximum point in the groups of 31–40 and 41–50 years in both sexes.

3.2.7. Infection according to season

Seasonality peaks in June (Fig. 6B), markedly in coastal provinces, lower in the highland, and less evident in the remaining zones (Fig. 6C).

The proportions of female patients were significantly higher than those of male patients within each month the whole year long, according to the *P* value (two-sided) obtained with the Fisher's exact test (Table 5, Fig. 7B). This means that the behavior of females and males linked to infection sources do not change according to the season. Case number curves of females and males appear to evolve in a parallel manner. No significant differences have been detected in the comparison between the month groups (Chi square test, $P = 0.93$), although the data show the lowest infection rates in December and January and the infection peaks in the June–July period (Fig. 6B).

Monthly increase and decrease should be linked to two main factors: (i) seasonal climatic conditions defining lymnaeid snail vector population dynamics and (ii) seasonal growing/culture trends and consumption availability of the vegetables involved in human infection because of carrying infective metacercariae.

3.2.8. Infection according to provinces

An analysis similar to the aforementioned one was made to infer the differences between the proportions of female patients and male patients regarding provinces instead of months. Number of fascioliasis patients (N), proportion of infected patients (%), confidence interval (CI) (% 95) and significance of the comparison of proportions were computed according to sex and provinces (table not shown). No significant differences were detected between province groups ($P = 0.710$), although data showed, in both sexes, the highest proportions in the central province of Binh Dinh.

Proportions of fascioliasis in female patients were significantly higher than fascioliasis proportions in male patients in each province, with exception of only three provinces: Can Tho and Kien Giang located in the extreme south of Vietnam, and Dien Bien in the eastern extreme

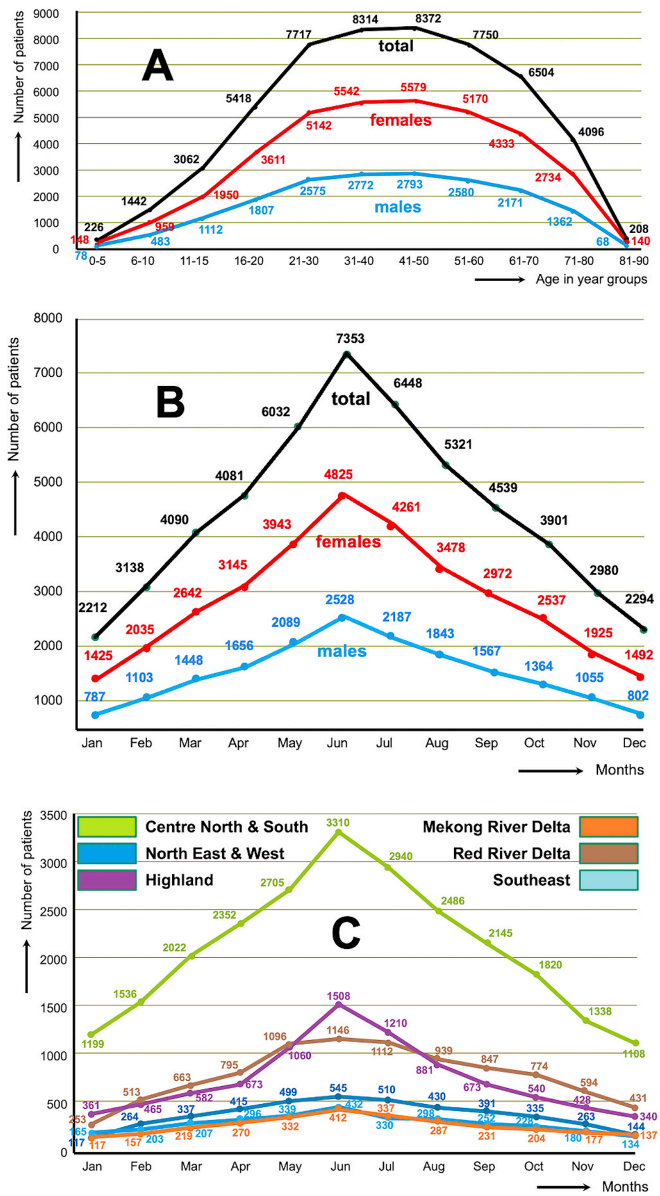

Fig. 6. Epidemiological characteristics of human fascioliasis in Vietnam. A) number of cases in total and after sex according to age groups; B) monthly seasonality of case numbers in total and after sex. C) monthly seasonality of case numbers according to the physiographic/climatic regions of the country.

north of the country. In these three provinces, the very low number of patients and proportions reflect the late arrival of the epidemic. In Can Tho and Dien Bien the first cases were detected in 2016, whereas in Kien Giang only in 2017. This highlights the importance of the geographical distance from the original focus in the speed of the disease spread (Fig. 7C).

3.3. *Correlations of province case numbers with risk indices*

The first analysis of the 11 provinces selected because of including meteorological stations and showing an initial gradual increasing yearly case number revealed that the number of cases is strongly seasonal and significantly related within time-to-events between seven and twelve months, which always included the nine-month-lag (Supplement 3). The correlation coefficients obtained when analyzing the epidemic period were very high, ranging between 0.36 and 0.63, including consistent results when considering whether only the endemic phase or the entire period (Table 6).

In the second analysis about a potential correlation between yearly province case number with yearly risk index values (instead of monthly values, as above), the results proved that the overall increase of yearly human fascioliasis cases do not correlate with climate change (Supplement 3).

3.4. *Nuclear rDNA and mtDNA markers of fasciolids*

The analysis of rDNA ITS-2 (365/364 bp in *F. hepatica* and *F. gigantica*, respectively) and ITS-1 (432 bp in both species), and the mtDNA *cox*1 (1533 bp) and *nad*1 (903 bp), showed that the sequences of the two mtDNA genes in all specimens belonged to *F. gigantica*. However, sequences of both *F. gigantica* and *F. hepatica* were found in the two ITS spacers in several specimens, with a clear predominance of *F. gigantica* (66%). In the remaining flukes, the detection of introgression proves that some flukes were hybrids (rDNA of *F. hepatica* and mtDNA of *F. gigantica*).

3.5. *Ribosomal DNA ITS-2 of Radix viridis*

The ITS-2 proved that lymnaeid populations belonging to *Radix viridis* were present in most of the samples surveyed throughout Vietnam. The analyses of sequences obtained from Ha Noi and Binh Din localities demonstrate that all these samples were identical, showing an ITS-2 sequence of 373 bp in length and 62.7% of GC content. This haplotype (LVT—H1) was compared with other GenBank sequences and an alignment was performed with other sequences/haplotypes showing at least >98% of similitude (according to BLASTN results).

Sequence alignment allowed us to verify that our haplotype

Table 4
Number of fascioliasis patients (N), proportion of infected patients (%), confidence interval (CI) (% 95) and significance of the comparison of proportions according to sex and age in Vietnam.

Age groups	Patients	Female patients		Male patients		P* value
years	Total	N	% (% 95 CI)	N	% (% 95 CI)	
0–5	226	148	65.49 (59.07 to 71.38)	78	34.51 (28.62 to 40.92)	≤ 0.001
6–10	1442	959	66.50 (64.02 to 68.89)	483	33.50 (32.11 to 35.97)	≤ 0.001
11–15	3062	1950	63.68 (61.96 to 65.37)	1112	36.32 (34.63 to 38.04)	≤ 0.001
16–20	5418	3611	66.65 (65.38 to 67.89)	1807	33.35 (32.11 to 34.62)	≤ 0.001
21–30	7717	5142	66.63 (65.57 to 67.68)	2575	33.37 (32.32 to 34.43)	≤ 0.001
31–40	8314	5542	66.66 (65.64 to 67.66)	2772	33.34 (32.37 to 34.36)	≤ 0.001
41–50	8372	5579	66.64 (65.62 to 67.64)	2793	33.36 (32.36 to 34.40)	≤ 0.001
51–60	7750	5170	66.71(65.65 to 67.75)	2580	33.29 (34.35 to 34.40)	≤ 0.001
61–70	6504	4333	66.62 (65.47 to 67.76)	2171	33.38 (32.24 to 34.53)	≤ 0.001
71–80	4096	2734	66.75 (65.29 to 68.18)	1362	33.25 (31.82 to 34.81)	≤ 0.001
81–90	208	140	67.31 (60.67 to 73.32)	68	32.69 (26.68 to 39.33)	≤ 0.001
P value**						0.30
Total	53,109	34,680	65.30 (64.89 to 65.70)	18,429	34.70 (34.29 to 35.11)	≤ 0.001

[*] analyzed with Fisher's exact test (two-sided).
[**] analyzed with Chi-square test; statistically significant when P < 0.05.

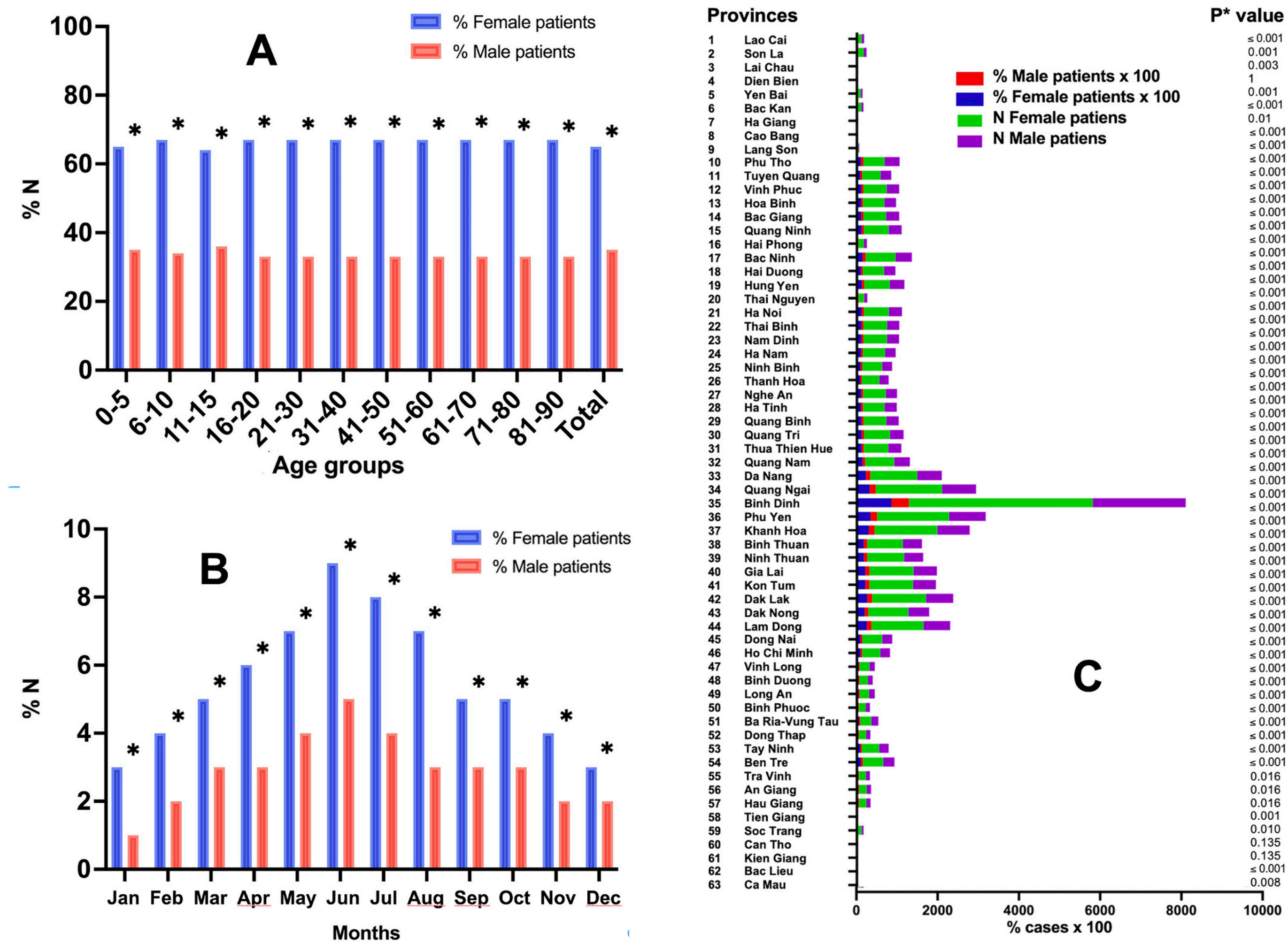

Fig. 7. Statistical analyses of the proportions of *Fasciola*-infected patients in Vietnam and their comparisons: A) According to sex and age groups in Vietnam. * = significant difference of sex proportions in each age group (Fisher's exact test); no significant differences have been detected in the comparison between the age groups (Chi square test, $P = 0.309$); B) According to sex and months in Vietnam. * = significant difference of sex proportions in each month (Fisher's exact test); no significant differences have been detected in the comparison between the months (Chi square test, $P = 0.930$); C) According to sex and provinces of Vietnam. * = P values (Fisher's exact test); no significant differences have been detected in the comparison between the provinces (Chi square test, $P = 0.710$). Bars are proportions of infected patients (% N); Fisher's exact test, two-sided; statistically significant when $P < 0.05$.

(LVT—H1) was identical to *Radix* (*Austropeplea*) *viridis* from Vietnam (GenBank Accession No. KF042387), differing in only two mutations (C/T in position 81 and T/A in position 382) and in one deletion (A/− in position 294), from *Radix* (*Orientogalba*) *viridis* specimen SNSD:Moll: S6832 (GenBank Accession No. LT220500). A microsatellite region was detected between positions 87 and 114 of the ITS-2 alignment, allowing for differences in length of ITS-2 sequences. The lack of some microsatellite repeats inside this region appears evident when comparing our haplotype with *Radix* (*A.*) *viridis* (GenBank Accession No. LT220499). Other sporadic mutations in the ITS-2 sequence in addition to differences in microsatellite repetitions, and more specifically differences in a poly "A" region at the 3′ end of the ITS-2, clearly evidence the existence of an intraspecific variability in the species *R. viridis*.

3.6. Disease spreading roles of lymnaeids and irrigated crop fields

In Vietnam, *R. viridis* snails show a marked amphibious eco-ethology, living specimens being frequently found on mud (Fig. 8A). Populations of *R. viridis* are very frequent in crop fields needing intense artificial irrigation, in which these snails are easily found in the field borders, even close to human dwellings (Fig. 8B). In that sense, the anthropophilic behavior of *R. viridis* is similar to that of the main fascioliasis vector *Galba truncatula* and explains its role in the transmission to humans.

Field surveys in the peri-urban and rural areas areas of Quy Nhon in Binh Dinh and Da Nang in the central coast but also in the peri-urban areas of the capital Ha Noi, showed that the populations of *R. viridis* are usual inhabitants in rice paddies, water spinach and other vegetable growing fields, sometimes ones besides the others along vast extensions (Fig. 8C). Indeed, rice and water spinach or water morning glory ("rau muong" in Vietnamese) are first and second vegetables in human diet in Vietnam. There are other vegetables traditionally included in the Vietnamese diet and whose ecological characteristics make them suitable as carriers of fasciolid metacercariae and hence sources for human infection, such as coriander ("ngoa"), watercress ("caûi xoong"), common basil ("rau ñaéng") and flagrant knotweed ("rau raêm").

The man-made and/or artificial irrigation canals used for the irrigation of these crop fields furnish, moreover, a very convenient way for lymnaeids to jump from one field not only to the neighboring one, but also fields located far away but depending on the same irrigation system (Fig. 8D).

Table 5
Number of fascioliasis patients (N), proportion of infected patients (%), confidence interval (CI) (% 95) and significance of the comparison of proportions according to sex and months in Vietnam.

Months	Female patients		Male patients		P* value
	N	% (% 95 CI)	N	% (% 95 CI)	
January	1425	2.68 (2.54 to 2.82)	787	1.48 (1.38 to 1.58)	≤ 0.001
February	2035	3.86 (3.67 to 3.99)	1103	2.07 (1.95 to 2.20)	≤ 0.001
March	2642	4.97 (4.79 to 5.16)	1448	2.72 (2.59 to 2.86)	≤ 0.001
April	3145	5.92 (5.72 to 6.12)	1656	3.11 (2.97 to 3.27)	≤ 0.001
May	3943	7.42 (7.20 to 7.64)	2089	3.93 (3.77 to 4.10)	≤ 0.001
June	4825	9.08 (8.84 to 9.33)	2528	4.76 (4.58 to 4.94)	≤ 0.001
July	4261	8.02 (7.79 to 8.25)	2187	4.11 (3.95 to 4.29)	≤ 0.001
August	3478	6.54 (6.34 to 6.76)	1843	3.47 (3.31 to 3.62)	≤ 0.001
September	2972	5.40 (5.40 to 5.79)	1567	2.95 (2.80 to 3.09)	≤ 0.001
October	2537	4.77 (4.59 to 4.96)	1364	2.57 (2.44 to 2.71)	≤ 0.001
November	1925	3.62 (3.46 to 3.78)	1055	1.98 (1.87 to 2.10)	≤ 0.001
December	1492	2.80 (2.67 to 2.95)	802	1.51 (1.40 to 1.61)	≤ 0.001
P value**					0.93
Total	34,680	65.29 (64.89 to 65.70)	18,429	34.70 (34.29 to 35.10)	≤ 0.001

* analyzed with Fisher's exact test (two-sided).
** analyzed with Chi-square test; statistically significant when $P < 0.05$.

Table 6
Analysis of the correlation between monthly risk indices and monthly human cases in the eleven Vietnamese provinces including meteorological stations and showing an initial gradual increasing yearly case number.

Provinces		Whole 1995–2019 period				Endemic period only			
A	B	C	D	E	F	G	H	I	J
–	General model	1995–2019	9	<0.001	0.13	–	–	–	–
17	Bac Ninh	2002–2014	11	<0.001	0.40	2008	11	<0.001	0.63
18	Hai Duong	2005–2019	11	<0.001	0.42	2013	11	0.005	0.37
23	Nam Dinh	2003–2019	12	<0.001	0.52	2015	12	<0.001	0.59
26	Thanh Hoa	2003–2019	11	<0.001	0.36	2014	11	0.009	0.51
27	Nghe An	2001–2019	10	<0.001	0.29	2013	10	0.012	0.42
29	Quang Binh	2002–2019	9	<0.001	0.36	2014	8	<0.001	0.52
33	Da Nang	1995–2019	8	0.002	0.25	2007	8	<0.001	0.45
35	Binh Dinh	1996–2019	7	0.054	0.13	2013	6	<0.001	0.45
37	Khanh Hoa	1996–2019	7	<0.001	0.24	2008	7	<0.001	0.36
42	Dak Lak	1996–2019	12	<0.001	0.51	2008	11	<0.001	0.53
46	TP. Ho Chi Minh	1996–2019	9	0.002	0.24	2008	9	0.002	0.41

A: number in map of Fig. 1; B: province name; C: year period assessed; D: time-to-event (time-lag in months) with coefficient of stronger magnitude; E: P value. F: correlation's R^2; G: year from which a stabilized plateau was reached (endemic phase); H: time-lag (in months) during the endemic phase with coefficient of stronger magnitude; I: endemic phase P value; J: endemic phase correlation's R^2.

3.7. Vegetable city markets

The high proportion of fascioliasis patients living in urban environments of Vietnam, including big cities, should be highlighted. The importance of fresh vegetables in human diet leads to plant collection in peri-urban and rural areas, their subsequent transport and ultimate selling in uncontrolled city markets (Fig. 8E). The emergence of fascioliasis in a country where fascioliasis never posed public health problems in the past, has therefore given rise to an unexpected, never previewed problem to a traditional vegetable distribution practiced throughout all provinces.

3.8. Livestock grazing and movements

Field surveys of transmission foci showed that the main domestic animal reservoir species involved in fascioliasis transmission is cattle, and secondarily buffaloes. Vietnamese farmers allow these ruminants to graze in harvested rice paddies where lymnaeid populations are active during irrigation and also afterwards thanks to the amphibious characteristics of *R. viridis* (Fig. 8F). In that way, these large ruminants play an important contribution to fascioliasis transmission by ensuring the liver fluke to close its life cycle in these crop fields. So, the Vietnamese tradition of combining vegetable production with livestock production offers fascioliasis the perfect way for its transmission.

Food demand for a markedly growing human population, and Vietnamese preference for fresh vegetables whose growing requires repeated irrigation, underlies the vast extensions of rice paddies found throughout the country. The intense movements of mainly cattle (Fig. 8G) but also buffaloes across provinces throughout the whole country were crucial in the spread of the disease.

4. Discussion

4.1. Magnitude of the outbreak

The magnitude of the spreading outbreak including over 53,000 subjects infected by *F. gigantica* up to cover all the 63 provinces of Vietnam in human fascioliasis is appreciated when considering that only 2500 cases, almost all by *F. hepatica*, were globally reported throughout 1970–1990 [17].

The fact that the human fascioliasis outbreak started in an initial geographical focus and subsequently spread up to cover all the provinces of such a latitudinally extended long country as Vietnam has no precedent. The two big human outbreaks (around 10,000 cases in 1989 and 5000 cases ten years later) occurred in Guilan, Iran, did not spread geographically [67,68].

Nothing suggested that a freshwater snail-borne, zoonotic and food/water-borne disease such as fascioliasis could follow a geographical spread until covering the whole country of Vietnam, because:

- At that time *F. gigantica* was considered a secondary causal agent

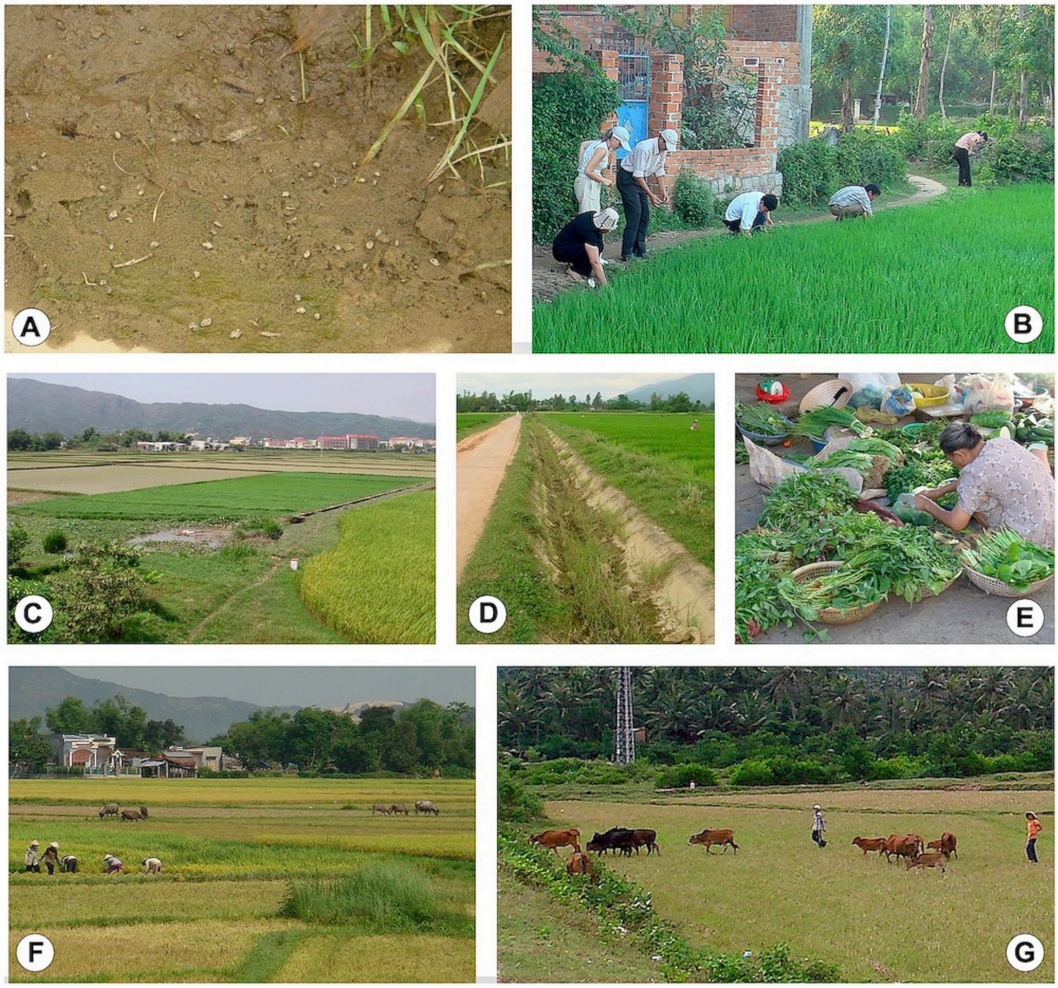

Fig. 8. Field studies of fascioliasis transmission foci in Vietnam: A) Specimens of *Radix viridis* snail vector species on mud; the amphibious behavior of this lymnaeid species allows it for a wide and fast geographical spreading capacity by passive transport when in mud attached to the hooves of moving animals, mainly human-guided livestock; B) Distribution of *R. viridis* populations along rice paddies; specimens of this snail have an ideal habitat in the irrigated rice fields, in whose borders active populations are usually found; its presence close to human dwellings speaks about its anthropophilia and role in the disease transmission to humans; C) Transmission foci in peri-urban areas; wide crop fields for the growing of rice and water spinach organized around artificial irrigation systems are frequently found in peri-urban areas; the vast rice culture extensions throughout Vietnam enable for the wide spread and distribution of *R. viridis*; D) Lymnaeids along irrigation canals; the very long man-made irrigation canals facilitate the spread of *R. viridis* throughout wide areas when swept away by the water current; populations of this lymnaeid are usually found along such irrigation canals; E) Freshwater vegetables sold in uncontrolled city markets; fresh vegetables are an important component of the traditional human diet in Vietnam; such city markets offering fresh plants collected in rural and peri-urban areas, whose growth needs frequent irrigation, are numerous in urban areas and underlie human infection of inhabitants living in cities and towns; water spinach or morning glory, the second vegetable in Vietnamese diet after rice, and other vegetables are sold in all these markets; F) Large ruminants grazing in harvested crop fields; cattle and buffaloes are traditionally allowed to graze in harvest crop fields, their stools therefore furnishing the needed fasciolid egg shedding for the subsequent infection of the lymnaeid snail vectors inhabiting these irrigated crop fields, thus closing the liver fluke cycle; G) Traditional ruminant moving; thousands of large ruminants, mainly cattle, are moved across provinces throughout the whole country every year, whether for slaughtering or raising in other places; these animal moving events contribute to the spread of both high liver fluke infection rates and *R. viridis* snails, as well as for the spread of viable fasciolid hybrids. Photographs S. Mas-Coma.

of human fascioliasis giving rise to only sporadic human infections worldwide.

- The cycle of this trematode around 14–23 weeks to complete all its development phases [69].

- The transmission of this disease needs that the larval stages of the fasciolid develop and asexually multiply in a freshwater snail and there is, therefore, no possibility for a fast direct transmission from human to human.

- The fasciolid life cycle is strongly influenced by climate factors [33], a crucial aspect which was a priori ruling out the possibility of a geographical spread of this disease throughout such a climatologically heterogeneous country as Vietnam.

All these aspects explain the long time that fasciolids require to geographically expand. Indeed, thousand-year long human-guided movements of domesticated pack animals along highly used routes were needed for the fasciolids to spread from the original Neolithic Near East Asia until covering the whole Asian continent [6]. The slow speed of geographical spread of fascioliasis can explain the relative long period from 1995 to 2019 that was required for the disease to cover the whole of Vietnam, even though this is a priori too fast within a frame of normal conditions.

In its turn, this also explains why the reaction by the health authorities of Vietnam followed progressive steps ahead in the epidemiological analysis and the design of control measures, as a reflection of how the disease outbreak was evolving.

In front of the significant space-time clusters which indicated an underlying cause different than chance, efforts were made to elucidate the cause(s) underlying such a spreading outbreak.

4.2. Discarding climate change as the cause of the outbreak

The results of the climate analyses provide crucial information for the appropriate One Health assessment [9]. On one side, the seasonality of human infections is supported by the significant correlation between monthly province case number and monthly risk index values, with a time-to-event variation range of 7–12 months depending on provinces (countrywide optimum average of 9 months). This variation range is potentially linked to (i) delays in individual case diagnosis, (ii) temporary combination of surface freshwater availability (enabling lymnaeid snails vector presence) by rainfall and vegetable crop artificial irrigation, and (iii) local meteorological differences of provinces according to the country physiographic/climatic zones. We already proved that the fascioliasis risk indices work appropriately in another monsoon country such as Pakistan, where we showed how the human infection waves induced by climate and irrigation occur independently one another [11].

On the other side, yearly case/risk index correlation analyses prove that the epidemic should not be attributed to climatic influences. Correlation disagreements between yearly case number and yearly risk index values in most provinces do not support the countrywide spread of human fascioliasis to be caused by climate change impact. This conclusion should be highlighted, because one of the main questions posed by the spread of a disease, as strongly influenced by climate factors such as fascioliasis [33,70], was how to understand an expansion up to cover the whole geography of a country as climatologically heterogeneous as Vietnam.

4.3. Discarding confounders as causes for the 2006 and 2009 rebounds

All confounders other than climate change were also discarded, as noted in Supplement 1 and related references [71–74].

4.4. Increased awareness and diagnostic training as rebound causes

After discarding the aforementioned confounders (Supplement 1), the conclusion is reached that only an increase of patient detection, as the consequence of improvements in the physician awareness and diagnostic capacity, was the cause of the two case number rebounds in question.

Regarding the 2006 rebound, interviewed Vietnamese health professionals openly attributed the strong awareness of fascioliasis emergence achieved among hospital physicians countrywide, to the diffusion of a book. This was a monographic book on human liver flukes written by the first and third authors of the present article and published in Vietnamese language in Ha Noi in 2004 [75]. The writing process was undertaken after the wake-up call represented by the hundreds of fascioliasis cases diagnosed and treated in the National Institute of Malariology, Parasitology and Entomology of Ha Noi (NIMPE) consulting-room from 2003 onwards. This book described fascioliasis and allowed for its differentiation from the other two liver trematodiases caused by *C. sinensis* and *O. viverrini* in Vietnam, and from the intestinal infection by another fasciolid such as *F. buski*, also present in Vietnam and whose eggs of similar morphology appearing in stools of the patients may lead to diagnostic confusion. This book was quickly diffused countrywide throughout hospitals, physicians and public health personnel. This proved to be a pragmatic step ahead for the quick dissemination of the needed baseline knowledge on fascioliasis, by solving the big problem posed by the language and additionally referring to the need to differentiate from the locally well-known fish-borne clonorchiasis and opisthorchiasis [76–78]. Moreover, the positive impact of this book was complemented with an intense additional diffusion activity by the first author (NVD) by giving oral lectures on fascioliasis and the outbreak in selected key hospitals of the country.

Similarly, the 2009 case rebound appears to be the consequence of the impact of the mission of staff experts of the WHO Collaborating Centre on Fascioliasis and Its Snail Vectors of Valencia (WHO CC SPA-37 and SPA-53) and responsible staff of the Department of Control of NTDs of WHO (Headquarters, Geneva) to Vietnam from 21 March to 11 April 2007. The main objectives of this mission were (i) to assess of the at-that-time unexpected question marks posed by the spreading outbreak of human fascioliasis and (ii) to evaluate the serological test being used by the Vietnamese public health professionals in these first years of outbreak.

To validate the serological test, fecal and serum samples from 94 patients originated from 21 provinces of Vietnam were provided by NIMPE Ha Noi, IMPE Quy Nhon and some provincial hospitals. The evaluation was made by comparison with previously validated diagnostic tests, including (i) the ELISA cathepsin L1 (CL1) test [79,80], (ii) the capture ELISA MM3-SERO test [81,82], (iii) the coprodiagnosis monoclonal antibody MM3 test [81,83], and (iv) the coprological Kato-Katz test. The diagnostic analyses of the human samples were made in the laboratories of the NIMPE (Ha Noi), IMPE (Quy Nhon), and (iii) Da Nang hospital (Da Nang) and used for the laboratory training of the Vietnamese technical staffs. The phenotypic characteristics of fasciolid eggs from Vietnamese patients were already published [84].

Additionally, WHO experts performed field surveys for the understanding of the disease transmission, included different multidisciplinary objectives detailed in Supplement 4 and related references [5, 9, 26, 29, 85, 86].

The time-to-event impact in both rebounds is of 2 years and suggests that *Fasciola*-infected people might have been overlooked or misdiagnosed before. The consequent impact on general physician awareness and diagnostic capacity improvement of the technical staff demonstrates the importance of such knowledge transfer and capacity building initiatives when dealing on emerging diseases about which local health professionals lack sufficient skills. Unnecessary surgery and/or radiotherapy applied to numerous cancer-misdiagnosed patients, in whom imaging techniques showed the presence of a liver tumor, evidences the importance of physician awareness about fascioliasis [87]. Indeed, a specific fascioliasis treatment with triclabendazole would have been sufficient to cure these patients. This remembers similar diagnosis confusions in Argentina in which liver-fluke infected patients were surgically intervened because of lithiasis suspicion [28].

4.5. Facilitating patient detection and treatment

The alarm caused by the report of hundreds of patients infected by *Fasciola*, at the end of the 1990s and beginning of the 2000s, led Vietnamese health authorities to raise the problem posed by the treatment of fascioliasis, which does not respond to praziquantel. WHO helped in obtaining a set of 1000 tablets of triclabendazole in 2003. Subsequently, the Vietnamese Ministry of Health requested the opportune evaluation of the safety and efficacy of this drug against the fasciolid infections of Vietnamese citizens. This evaluation study was performed, the results reported to WHO and published in 2005 [88], and the final certificate granted by the Vietnamese Ministry of Science and Technology in 2006. This initial step allowed NIMPE and WHO to go for the request of the corresponding triclabendazole tablet donation and in that way assure the countrywide coverage and needs, and enable for the subsequent inland distribution by NIMPE to all hospitals of Vietnam.

This was complemented by the use of radio broadcasting to widely disseminate information on the outbreak of fascioliasis, notably among the inhabitants of the rural areas where infection risk is higher, but also into urban areas where risky vegetables from neighboring rural zones are traditionally sold in uncontrolled city markets. Radio diffusion referred to typical fascioliasis symptoms and urged persons presenting with such symptoms to go to the closest hospital for their costless diagnostic and treatment.

4.6. Triclabendazole treatments

A total of 68,000 tablets have been made available by WHO Headquarters Geneva to the health authorities of Vietnam since 2006, within the WHO programme for triclabendazole donation [89] (Supplement 4). Until 2019, treatments needed to cover the countrywide outbreak included shipments in 2006 (20,000 tablets), 2007 (10,000 tablets), 2008 (8000 tablets), 2011 (5000 tablets), 2014 (10,000 tablets), and 2017 (15,000 tablets). These quantities of tablets were applied to treat the total of 49,657 patients diagnosed between 2006 and 2019. It shall be considered that the shelf life of triclabendazole is three years.

4.7. Follow-up of the fascioliasis outbreak

The great positive impact of the aforementioned WHO mission, the active collaboration during the mission, and the successful implementation of knowledge transfer, laboratory training and field co-work, led the government of Vietnam to support research on fascioliasis in the country. Subsequently, an official delegation of Vietnam was organized to visit the WHO CC in Valencia in 2009 to prepare: (i) the future joint collaboration on epidemiology, diagnosis and training, and (ii) a plan for controlling fascioliasis in Vietnam.

Accordingly, the first and last authors of this article (NVD, SMC) launched a joint follow-up and study of the evolution of the human fascioliasis countrywide outbreak from the year 2011 [90]. The COVID-19 pandemic, started in southern Chinese at the end of 2019 and subsequently expanding globally from 2020, led both authors to decide to compile the total data accumulated since 1995 up to 2019 and to proceed with the corresponding publication of their multidisciplinary analysis and conclusions, by taking into account that (i) the fascioliasis outbreak had already covered all provinces of the country, (ii) the coronavirus pandemic represented a risk regarding its potential to disturb hospital services and their reporting capacities, and (iii) this publication may provide useful information for One Health initiatives to be launched in countries of South and South East Asia where human fascioliasis reports by *F. gigantica* are at present increasing [13].

4.8. Incidence per provinces

The only country where human incidences are known from appropriate assessment studies is France, due to the importance of this disease having been recognized in that country already since mid-last century. However, human fascioliasis incidence values in France were indirectly estimated from local prevalence data in different departments of the country and not from real number of new cases diagnosed per year [91]. The incidence in the 95.7 million 2019-population of Vietnam is higher than 10 times that of the only so far available incidence of 0.61/100,000-year by *F. hepatica* in the 64.4 million population of France in similar years [91].

The reduced fluctuations of the yearly case numbers along the endemic phase inside each of the 47 provinces markedly differs from the endemic situation reported about 616 persons diagnosed to be infected by liver flukes in the Limousin region of France along the 1955–1998 period, including irregular strong fluctuations varying between 2 and 80 cases/year and with peaks in 1958, 1963, 1968, 1969, 1971, 1977, 1981 and 1987, and final decrease down to two cases in 1998 [92]. In the French Limousin, this yearly incidence irregularity of human fascioliasis is related to differences in climatic conditions, i.e. mainly rainfall, throughout the different years analyzed. In that sense, the scarce yearly oscillations of new cases/year/province in Vietnam are similar to those reported from the province of Guilan in Iran along the 2006–2014 period [30].

In Vietnam, the overall scenario indicates that fascioliasis transmission and human infection through locally produced vegetables mainly depends on artificial irrigation, which should act buffering changing climate and precipitation influences.

4.9. Infection according to sex, age and season

The higher infection rates in females agree with human endemic areas of Bolivia [24], Peru [29], and Egypt [26]. The greater affection of women and girls in rural areas is known to be related to their involvement in food preparation and work in the kitchen, as verified in other endemic countries [5,8]. In Vietnam, however, the proportion of infected women is markedly higher than in men in all age groups at a level which exceeds the sex differences in endemic areas of other countries [5,24,26].

In Vietnam, child infection rates, including very early infections [93], are worrying. Children are known to be underdiagnosed in cases of low or non-permanent symptoms, which are easily given no importance by parents [5]. When overlooked, chronicity may last for years. The age distribution in Vietnam differs from that in Bolivia [24], Peru [29], and Egypt [26], where children are the most affected. The lack of differences between the age groups contrasts with the maximum values described in children between 9 and 11 years in other human hyperendemic areas [5,24,26].

The explanation of the lack of infection differences between age groups observed in Vietnam may be related to the different diagnostic technique. Such observations in other countries were based on infection rates obtained by coprological diagnosis in surveys, whereas in Vietnam diagnosis has been made by serology applied to patients attending health centers. Moreover, serological tests allow for an early detection from 2 to 3 months post-infection (whereas coprological analyses only from 3 to 4-month post-infection) and have a higher capacity to detect infection at very low burdens [16].

Seasonality patterns fit the climatic characteristics of each country zone. Vietnam is located in the monsoon region and deeply influenced by the sea, including rainy and dry seasons. In the coastal region and highlands, January–July is appropriate for crop cultures needing high irrigation and vegetable consumption is higher. Vegetables are less consumed in the Mekong Delta and inversely in the North [94]. Cattle prevalence is also higher in the rainy than in the dry season [95].

4.10. Molecular characterization of the causal agent and hybrid detection

The first molecular assessment of the causal agent by DNA marker sequencing demonstrated that *F. gigantica* was involved, even in patients presenting with ectopic infection [96]. This was in agreement with the aforementioned historical records indicating that it was this fasciolid species the one to underlie the fascioliasis endemicity in the livestock of Vietnam since old times. A subsequent study allowed for the detection of *F. gigantica/F. hepatica* hybrid worms infecting both livestock and humans in Vietnam (localities not specified) already from the year 2001 [97].

Our sequencing of fasciolids infecting cattle in the provinces of Binh Dinh and Da Nang, obtained during the 2007 WHO mission, also proved that the majority were genetically "pure" *F. gigantica* flukes. Admixed and introgressed hybrid forms were accompanying this majority of "pure" *F. gigantica* flukes. The clear predominance of *F. gigantica* (66%) in admixed fasciolid specimens presenting both *F. gigantica* and *F. hepatica* within the nuclear rDNA operon also fits. All in all, these results agree with historical data indicating that *F. gigantica* is the original fasciolid in Vietnam, as well as throughout southeastern Asia [6].

Regarding the fasciolid hybrids detected during the WHO mission, the introduction of *F. hepatica* with imported livestock from other countries endemic of *F. hepatica* should have occurred before the year 2007. Indeed, introgressed hybrids were already found in flukes infecting cattle and buffaloes, as well as human patients, in Vietnam from 2001 [97]. Although unfortunately the specific localities where such hybrids were found were not detailed, these results already proved that *F. hepatica* was introduced earlier into the country. Indeed, *F. hepatica* had never been reported in Vietnam before.

The existence of the two seaports of Quy Nhon and Da Nang in this central part of Vietnam, through which livestock importation from other countries is carried out, is the only conceivable local door of entrance of *F. hepatica*. Although countries exporting livestock ensure that they only export healthy and treated animals, liver fluke infection is not a priority in international rules because fascioliasis is already almost everywhere in animals [6]. Consequently, in developing countries such as those in southern and southeastern Asia, animal quarantine measures are usually not applied to importation practices, because of insufficient manpower and funds for such control measures [98]. So, liver fluke infection is not looked for in the freely released imported animals, nor are those animals preventively treated against fascioliasis before being added to local herds, according to the traditional veterinary fattening practices for the recovery and weight gain of the imported animals after a long sea trip [98,99]. This should have enabled the foreign *F. hepatica* to encounter native *F. gigantica* inside the liver of the freely grazing imported animals, with subsequent fluke cross-breeding leading to rDNA mixing and mtDNA introgression. Aspects agreeing with this are (i) the high seroprevalence and egg shedding found in calves as young as 3 to 4 months old, i.e. young animals to become infected soon after birth [100], which indicates a very high infection risk in Binh Dinh, (ii) the presence of *R. viridis* snails throughout fields used for livestock grazing in this province (Fig. 8G), and (iii) the experimentally verified capacity of *R. viridis* to transmit both fasciolid species [101,102] and in that way enable for the local transmission of the newly emerging hybrid lineages.

These findings initially led to speculate about the possibility that a hybridization phenomenon generating hybrids of high spreading capacity in the central coast of Vietnam could be the responsible for the countrywide outbreak. Our subsequent studies indicated that this was not the case and that hybrid flukes were simply accompanying the dominant “pure” *F. gigantica* along the same spreading trend [97]. This conclusion was further verified when sequencing more numerous fasciolid specimens from many geographical origins in Vietnam many years later [72].

All in all, this proves that livestock was imported through the seaports and hence livestock populations were growing in central Vietnam at the beginning of, or perhaps even sometime before, the starting of the fascioliasis countrywide epidemic here in question. This is a key point to consider, given that the epidemic started in, and spread from, this central coastal zone of Vietnam.

Hybrid detection proved, however, to be very useful in the assessment of the original geographical focus of the outbreak and to conclude that livestock importation by maritime way from other countries have had a role in the origin of the epidemic in the central coast of the country.

4.11. Ribosomal DNA ITS-2 of Radix viridis

Radix viridis is a small species widely distributed throughout southeastern Asia and the Far East [6]. When comparing the *ITS-2* sequences found in the *R. viridis* specimens that we collected in Vietnam with those available in the GenBank [44,103–106], it may easily be concluded that this radicine lymnaeid vector species shows a wide intraspecific variability of the rDNA ITS-2.

Consequently, the haplotype uniformity of the specimens from Ha Noi and Binh Din localities indicates a recent spread inside Vietnam, a phenomenon which fits the recent very numerous human-guided movements of cattle from central Vietnam to the rest of the country [71,73,74]. This means that *R. viridis* specimens were passively transported when in mud attached to the hooves of the moved animals, as well known in fascioliasis spread [6].

4.12. Lymnaeid role in disease transmission and spread

The lymnaeid snail vectors, their ecology and ethology, mainly the amphibiousness and population dynamics, are crucial in defining the phases of the geographical spread of fascioliasis:

- Initial dissemination phase: This phase corresponds to the initial geographic expansion, whose duration varies depending on the potential habitats for the lymnaeids. Numerous and closer freshwater habitats facilitate a faster lymnaeid dissemination by livestock.
- Epidemic phase: The speed and length of this phase relies on vegetable consumption, livestock management (continual livestock seesawing releasing eggs leading to higher lymnaeid infection rates), and surface water availability (natural rainfall, artificial irrigation of crop fields). Several epidemics have been reported in different countries, including from hundreds to thousands of patients, although they always occurred locally and never spread geographically [107].
- Endemic phase: The intensity of the endemic final plateau additionally depends on the local environment (abundance of transmission foci) and climatic conditions, from scarce interannual variation as in rice paddies of Vietnam or high-altitude permanent foci of the Bolivian Altiplano [24], to pronounced annual ups and downs due to interannual rainfall differences in France [92], or intermediate situations in Iran [30].

The capacity of *R. viridis* to transmit *F. hepatica* was already highlighted in Japan [101] and Korea [102]. This means that Vietnamese *R. viridis* is able to transmit both *F. gigantica* and *F. hepatica* and is therefore an ideal transmitter of fasciolid hybrids and should have facilitated the origin, establishment and subsequent spread of such hybrids.

Moreover, its amphibious behavior enables it to be an efficient disease spreader by surviving in mud attached to the hooves of moving animals.

4.13. Irrigated crop fields and vegetable city markets

The importance of cultures of rice and water spinach or water morning glory (“rau muong” in Vietnamese) as transmission foci of fascioliasis in other endemic countries is well known [8].

The high proportion of fascioliasis patients living in urban environments of Vietnam, including big cities, appears to be higher than in other countries where urban fascioliasis is known to occur [8]. It should here be considered that fasciolid metacercariae keep their infectivity up to seven months and sometimes even longer when under appropriate conditions [108], and are, therefore, able to survive the transport from the rural area to the city market and keep their infectivity until the vegetable carrier is sold.

The proportions of patients living in rural/urban areas and activities related to vegetables/livestock fit this epidemiological scenario.

4.14. Main confluent factors underlying the human fascioliasis spread

Our multidisciplinary analysis has shown that many confluent factors underlie human fascioliasis spread from 1995, in a country without previous public health problems due to this disease.

Between 1986 and 2017, the human population grew from 60 million to nearly 90 million, with a rural population of 62 million [109], leading to the need for increasing food production. Livestock proved to be a highly dynamic sector along 1995–2019, with great zonal differences. The growth of animal and crop production coevolved. Rice culture expansion provided the context for livestock production and today represents 90% of cereal resources [109]. In fascioliasis, it is for human-guided livestock to spread both fasciolids and lymnaeid vectors [6]. Whereas buffalo populations slightly decreased, cattle increased from 3.6 million in 1990 to 6.7 million in 2006 and 5.2 million in 2019. Large ruminant movements are yearly constant events inside Vietnam involving many thousands of mainly cattle and secondarily buffaloes, mostly for slaughtering but also for raising in other places [73].

The intense movements of mainly cattle (Fig. 8G) but also buffaloes across provinces throughout the whole country, involving many thousands of animals along a year period and which are known to have been widely and repeatedly practiced during the 1995–2019-year period [73], were crucial in the spread of the disease.

To get an idea of the magnitude of livestock movements throughout Vietnam during the period in question, one has only to consider that a total of 29,129 animal movement events involving 1,064,365 animals (11,417 buffaloes, 54,573 cattle and 998,375 pigs) were recorded in eleven provinces along a very short time such as that from March to August 2014 [74]. These animals were moved out from >200 districts of central provinces to 45 provinces throughout Vietnam. Movement events and number of each livestock species moved were relatively constant throughout the study period. South-Central provinces proved to move animals in larger numbers compared with North-Central provinces. In the deltas, family farms develop cattle fattening activities, by benefitting from the considerable rice straw. In the South, rapid urbanization led to livestock concentration zones and system intensification, whereas in the North family farm dairy production underlies slower processes. The proportion of moved animals was 51% for slaughtering, 38% for raising in other places, and 11% for other purposes [74].

Such an evolving scenario fits the countrywide spread and is in line with our analyses ruling out all other potential confounders (Supplement 1). Highly infected cattle, moved from the central coastal provinces, has been the main spreader. Increases in temperature and rainfall (two factors favoring fasciolid transmission) [33] likely led to very high prevalences in cattle (87.2% seropositivity) [95] throughout the aforementioned coastal zones.

Moreover, there are extensive livestock movements throughout southern and south-eastern Asia. Large ruminants from India, Bangladesh, Myanmar, Thailand and Lao PDR are transported across country borders, mostly unofficially and without health control, with Vietnam being the main importer for its own market and as transit towards China through the northern border [71]. Whereas these countries are sources for *F. gigantica* introduction, cattle importation from Australia and USA [71] may have introduced *F. hepatica* and led to hybrid origin. Indeed, the central Vietnam seaports of Da Nang (Da Nang province) and Quy Nhon (Binh Dinh province) are foreign livestock receptors [https://www.australiaslivestockexporters.com/vn/export-services-vietnam.html]. Transborder-introduced goats, pigs and horses from China might have been also involved in the North [71]. Hybridization of recently-introduced *F. hepatica* with native *F. gigantica* likely occurred when imported animals are released for fattening and weight recovery after the trip. The molecular analyses of fasciolids indicate more than one foreign introduction event and more hybrids in central than in northern provinces [72].

On one side, these moving ruminants passively spread *R. viridis* snails and contributed to the increasing distribution of lymnaeid populations throughout all irrigated crop fields. On the other side, cattle and buffaloes infected by high liver fluke intensities [95] expanded such burdens from the central coastal provinces of initially Da Nang and Binh Dinh up to the southern and northern provinces until finally covering the whole country. Livestock imported in an uncontrolled way from other southern and southeastern Asian countries and introduced through the western borders in the way to China through the northern Vietnamese border [71], may have additionally contributed to the wide fascioliasis spread.

The snail *R. viridis* was crucial in both origin and spread. Our studies showed this lymnaeid collected in peri-urban and rural areas to be (i) markedly amphibious, allowing for its transport when in mud attached to ruminant hooves [6], (ii) distributed throughout rice paddies and vegetable cultures [94] and (iii) able to transmit *F. gigantica* and *F. hepatica* [101,102] and consequently also hybrids. The habit to use harvested crop fields for ruminant grazing assures transmission and high infection rates. Fresh vegetables traditionally sold in uncontrolled city markets underlie urban infection [8]. Past fascioliasis inexistence and consequent public ignorance led people to no precaution in vegetable consumption.

5. Conclusions

The very wide geographically spreading epidemic and endemic of human fascioliasis in Vietnam has no precedent and offers the framework for appropriate assessments of epidemiological aspects of this disease as never before. The present multidisciplinary and interdisciplinary analytical study is in agreement with the WHO recommendation to use One Health approaches [110] to achieve 2030 goals [111], within a global strategy also pushed by the World Organization for Animal Health (OIE-WAHO) [112] and the Food and Agriculture Organization (FAO) [113].

The following conclusions should be highlighted because of their interest to be globally extrapolated:

A) At human level:

– Fascioliasis is able to give rise to geographically spreading human outbreaks despite being a snail-vector borne, zoonotic and plant/waterborne disease.

– Human fascioliasis shows a spreading speed considerably slower than the speed of infectious diseases transmitted directly human-to-human.

– The high amount of cases demonstrates that *F. gigantica*, despite always being considered a secondary causal agent of human infection, is also able to cause epidemic and endemic situations similar to *F. hepatica*.

– The very big magnitude of the human fascioliasis outbreak in Vietnam is a wake-up call for south and southeastern countries of Asia which present the highest human population densities with increasing food demands, uncontrolled livestock inter-country exchange, foreign import practices, and monsoon's increasing climate change impact [13].

– The outbreak situation in Vietnam shows how human fascioliasis may reach highly worrying and surprisingly extreme epidemic and endemic rates in areas where children are not the most affected age group.

– In areas where local diet relies on a great proportion of vegetables potentially carrying metacercariae, there is the additional risk of urban fascioliasis due to uncontrolled city markets selling vegetables collected in crop fields from peri-urban and nearly rural zones.

B) At livestock reservoir level:

– Human-guided movements of livestock from an original area, where ruminant infection rates were very high, prove to be the way used by fasciolids and lymnaeid vectors to spread around and subsequently lead to the spread of human infections throughout all provinces of the country. This is a wake-up call for other countries in which such livestock intra- and intercountry exchange is practiced.

– Livestock management including the use of irrigated crop fields for livestock grazing prove to be an efficient way to spread and increase the transmission of the disease.

– Importing livestock from foreign exporting countries where the fasciolid species present differs from the one existing in the importing country is a practice of high risk regarding the potential introduction of foreign fasciolid strains and increasing fascioliasis rates, both prevalences and intensities, not only initially at the importing local areas but also subsequently to other areas where animals may be moved to.

C) At snail vector level:

– *R. viridis* is a highly efficient fascioliasis transmitter, even more than the so-far considered the best fascioliasis vector *Galba truncatula* [114], because *R. viridis* is able to transmit the two fasciolid species and consequently also hybrids.

– *R. viridis* is a lymnaeid species of marked anthropophilic etho-ecological characteristics similar to those of *G. truncatula*, in this way facilitating human infection.

– *R. viridis* is a lymnaeid species of marked amphibiousness and consequently a highly efficient fascioliasis spreader because of its great colonizing capacity when passively transported by attaching to the mud adhered to the hooves of livestock species.

– The adaptation capacity of *R. viridis* to different climatological zones of Vietnam underlies its ability to be passively transported to even very long distances and colonize extreme situations such as the Near East [44], western Europe [104] or Africa [115]. This is a wake-up call about a potential wide global spread of this efficient transmitter and the subsequent repercussions on the geographical spread and increasing transmission of this disease.

D) About transmission and epidemiological characteristics:

– Fascioliasis proves to have the capacity to give rise to a big spreading human outbreak without climate change involvement. Indeed, climate change has been highlighted as the main potential risk power for the spread of fascioliasis throughout the recent years.

– Altitude does not appear to be a decisive factor for the existence of high endemic situations of human fascioliasis, according to the higher transmission capacity enhanced at high altitude throughout the tropical and subtropical latitudes [116], after verifying that human fascioliasis in Vietnam spread throughout both coastal lowlands and mountainous highlands.

– Incidence is a crucial epidemiological factor which is appropriately obtained for human fascioliasis for the first time, thanks to the opportunity provided by the outbreak occurred in Vietnam.

– Logistic regression curves have been obtained for human fascioliasis for the first time when assessing the epidemiological patterns followed by the chronology of the annual number of cases per Vietnamese provinces, including varying curve slopes according the different zones of the country when moving away from the areas where the outbreak originated.

– Fasciolid hybrids proved to not be the cause of the spreading outbreak but only appear accompanying the geographical diffusion of the expanding fasciolids. Interestingly, fasciolid hybrids have shown to be useful markers for the follow-up of the epidemiology and spread of human fascioliasis.

E) For control:

– The time-to-event range of 7–12 months (average of 9 months) verified in the correlation of monthly province case number and monthly risk index values, obtained in deep analyses throughout many years, many provinces and different climatic zones, becomes a very useful tool for prevention and control of human fascioliasis. This tool may be useful in (i) areas suffering abnormal extreme climate situations such as catastrophic climate events, drastic rainfall changes, heat or cold waves, inundations, etc., and (ii) also in areas where climate factors follow a regular mono- or bi-seasonality pattern, as well as (iii) in areas suffering from multi-year cycling climate changes such as those of El Niño or La Niña phenomena.

– Information, education and communication to the public by radio broadcasting proved to be very helpful and efficient for leading symptomatic patients to hospital and health centers for early diagnosis and treatments. This strategy of passive detection of patients was later incorporated in the guideline list of human fascioliasis control strategies recommended and supported by WHO [117].

– Combining broadcasting leading to early diagnosis and costless treatment of patients thanks to the WHO triclabendazole donation programme proved to be a very useful strategy.

– General physician awareness and the improvement of the diagnostic capacity of the technical staff prove the successful impact of such knowledge transfer in facilitating and increasing patient infection detection, above all when dealing on emerging situations occurring in areas where local health professional lack sufficient skills and, as in the case of Vietnam, whose language poses difficulties in obtaining the adequate information.

Ethics statement

Patient data collection and analyses were approved by the following ethical committees of Vietnam and Spain, in agreement with the principles expressed in the Declaration of Helsinki: (i) the Institutional Review Board for Ethics in Biomedical Research, Ha Noi Medical University, Ministry of Health, Ha Noi, Vietnam (Approval No. 4018/HMUIRB, 26 December 2018); (ii) the Comité Etico de Investigación en Humanos de la Universidad de Valencia, Valencia, Spain (Approval No. H1496156195013, 1 Junio 2017).

Collection and analyses of flukes from animals and lymnaeid snail vectors from Vietnam, and related experimental research, by the members of the Valencia WHO Collaborating Centre in the Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain, were carried out under permission from the following ethical committees of Spain, strictly following the institution's guidelines based on the Directive 2010/63/EU: (i) Comité de Etica de Experimentación y Bienestar Animal de la Comisión de Etica de la Universidad de Valencia (Approval No. A1263915389140); (ii) Comité de Etica en Investigación Experimental de la Universidad de Valencia (Approval No. H132191024200); (iii) Ethics Animal Research Committee of Polytechnic University of Valencia (Approval No. P2–13–07–09); (iv) Servicio de Sanidad y Bienestar Animal, Dirección General de Producción Agraria y Ganadería, Consellería de Presidencia y Agricultura, Pesca, Alimentación y Agua, Generalitat Valenciana, Valencia, Spain (Approval No. 2015/VSC/PEA/00001 tipo 2).

Field collection of lymnaeid snail vectors in Vietnam did not need any specific permission because made in public land.

Consent for publication

All authors read and approved the final version for publication consideration.

Funding

Supported by National Foundation for Science & Technology Development in Vietnam (Grant No. 114/QD-HDQL-NAFOSTED). Spanish collaboration funded by: CIBER de Enfermedades Infecciosas Project CB21/13/00056, ISCIII, Ministry of Science and Education, Madrid, Spain; Projects No. 2016/099 and 2021/004 of the PROMETEO Program, Programa of Ayudas para Grupos de Investigación de Excelencia, Generalitat Valenciana, Valencia, Spain; and Project of the Marie Skłodowska-Curie Actions programme (Horizon Europe), Grant ID 101062347, EC, Brussels. The funders had no influence on the design or conduct of the study and were not involved in data collection or analysis, in the writing of the manuscript, or in the decision to submit it for publication.

CRediT authorship contribution statement

Nguyen Van De: Data curation, Methodology. **Pham Ngoc Minh:** Data curation, Investigation. **Thanh Hoa Le:** Data curation, Methodology. **Do Trung Dung:** Data curation, Methodology. **Tran Thanh Duong:** Data curation, Methodology. **Bui Van Tuan:** Data curation, Methodology. **Le Thanh Dong:** Data curation, Methodology. **Nguyen Van Vinh Chau:** Data curation, Methodology. **Pablo F. Cuervo:** Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **M. Dolores Bargues:** Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. **M. Adela Valero:** Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Albis Francesco Gabrielli:** Investigation, Writing – original draft, Writing – review & editing. **Antonio Montresor:** Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review & editing. **Santiago Mas-Coma:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Datasets generated for this study are available on request to the corresponding author.

Acknowledgements

Studies of this article have been performed within the framework of the global initiative of WHO against human fascioliasis (WHO Headquarters, Geneva, Switzerland). Authors acknowledge the collaboration by the staffs from WHO-WPRO Office and NIMPE in Ha Noi, IMPE in Quy Nhon and Ho Chi Minh Hospital of Tropical Diseases. Dr. John P. Dalton (Ireland) and Dr. Florencio M. Ubeira (Spain) are acknowledged for providing the appropriate diagnostic tests needed for the validation of the Vietnamese serological test for the diagnosis of human fascioliasis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2024.100869.

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