Multi-antigen serology and a diagnostic algorithm for the detection of arbovirus infections as novel tools for arbovirus preparedness in southeast Europe (MERMAIDS-ARBO): a prospective observational study

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Summary

Background Arboviruses are increasingly affecting Europe, partly due to the effects of climate change. This increase in range and impact emphasises the need to improve preparedness for emerging arboviral infections that often co-circulate and might have overlapping clinical syndromes. We aimed to strengthen surveillance networks for four clinically relevant arboviruses in southeast Europe.

Methods This study reports an in-depth analysis of the MERMAIDS-ARBO prospective observational study in adults (ie, aged ≥18 years) hospitalised with an arbovirus-compatible disease syndrome in 21 hospitals in seven countries in southeast Europe over four arbovirus seasons (May 1–Oct 31, 2016–19) to obtain arbovirus prevalence outcomes. The main objectives of the MERMAIDS-ARBO study, describing the clinical management and outcomes of four arboviruses endemic to southeast Europe, including Crimean–Congo haemorrhagic fever virus (CCHFV), tick-borne encephalitis virus (TBEV), Toscana virus, and West Nile virus (WNV), are reported elsewhere. In this analysis, given the challenges associated with arbovirus diagnostics, we developed a diagnostic algorithm accounting for serology outcomes and sample timing to study arbovirus prevalence in southeast Europe. Serum samples were collected on days 0, 7, 28, and 60 after hospital admission and tested for anti-CCHFV IgG and IgM antibodies with ELISAs (confirmed with an indirect immunofluorescence test) and for IgG and IgM antibodies specific to TBEV, Toscana virus, and WNV with custom-printed protein microarrays (confirmed with virus neutralisation tests). All acute-phase samples were tested by PCR for all four viruses. Descriptive analyses were performed for virus-reactive cases by geography and year, and possible factors (eg, age, sex, and insect bites) associated with virus reactivity were assessed.

Findings Of 2896 individuals screened, 913 were eligible for inclusion, of whom 863 (514 men, 332 women, and 17 unknown) had samples sent to the study reference laboratories and were included in molecular and serological analyses. Some individuals had insufficient clinical data to be included in the clinical analysis, but met the eligibility criteria for and were included here. Serum sampling was incomplete (eg, samples missing from one or more timepoints or no data on time since symptom onset) for 602 (70%) patients, and the timing of collection was often heterogeneous after symptom onset up to 40 days (average median delay of 5–6 days across all timepoints), affecting the ability to diagnose arbovirus infection by serology. By use of an interpretation table incorporating timing and completeness of sampling, one (<1%) participant had a confirmed recent infection with CCHFV, ten (1%) with TBEV, 40 (5%) with Toscana virus, and 52 (6%) with WNV. Most acute confirmed infections of Toscana virus were found in Albania (25 [63%] of 40), whereas WNV was primarily identified in Romania (36 [69%] of 52). Albania also had the highest overall Toscana virus seropositivity (168 [60%] of 282), mainly explained by patients confirmed to be exposed or previously exposed (104 [62%] of 168). Patients without antibodies to WNV or Toscana virus were significantly younger than patients with antibodies (mean difference –8·48 years [95% CI –12·31 to –4·64] for WNV, and –6·97 years [–9·59 to –4·35] for Toscana virus). We found higher odds of Toscana virus reactivity in men (odds ratio 1·56 [95% CI 1·15 to 2·11]; p=0·0055), WNV reactivity with mosquito bites versus no mosquito bites (2·47 [1·54 to 3·97]; p=0·0002), and TBEV reactivity with tick bites versus no tick bites (2·21 [1·19 to 4·11]; p=0·018).

Interpretation This study shows that despite incomplete and heterogeneous data, differential diagnosis of suspected arbovirus infections is possible, and the diagnostic interpretation algorithm we propose could potentially be used to

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For the Greek, Albanian, Romanian, Bosnian, Serbian, and Croatian translation of the summary see Online for appendix 1

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strengthen routine diagnostics in clinical settings in areas at risk for arboviral diseases. Our data highlight potential hotspots for arbovirus surveillance and risk factors associated with these particular arbovirus infections.

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Introduction

Vector-borne diseases, including arboviruses, are climate-sensitive infections increasing in geographical range and impact due to a complex set of drivers.[1–4] Climate change is highly correlated with changes in the abundances of reservoir hosts and vectors, as well as virus replication patterns, which could affect the epidemiology and distribution of arboviral diseases in regions where arboviruses are currently less prevalent, including Europe. Consequently, arboviruses are progressively expanding their co-circulation across these regions, underscoring the need to consider multiple arboviruses in differential diagnosis, even when the frequencies of arbovirus occurrences and co-occurrences are still rare. An additional complicating factor is that symptoms of arbovirus infections often overlap with those of sometimes more common infectious diseases, ranging from mild febrile illness to more severe conditions, such as neuroinvasive disease or severe haemorrhagic fever.[4]

The changing arbovirus distribution pattern, including increasing co-circulation, and challenges of symptom-based diagnosis highlight the need for syndromic multiplex diagnostic panels for preparedness and early identification of multiple arboviruses. However, laboratory diagnosis of arbovirus infections is challenging because viraemia is short-lived, and therefore the window for virus detection is limited to the acute phase of infection. Thus, a diagnosis of arbovirus infection is often based on serology, which is complicated by antibody cross-reactivity between closely related co-circulating arboviruses, such as the flaviviruses Usutu virus and

Research in context

Evidence before this study

We searched PubMed for articles published in English from database inception up to July 23, 2024, using the search terms “prevalence study”, “prevalence”, “circulation”, “cases”, “presence” OR “seroprevalence”, AND “Crimean–Congo haemorrhagic fever virus”, “CCHFV”, “Toscana virus”, “TOSV”, “tick-borne encephalitis virus”, “TBEV”, “West Nile virus”, OR “WNV”, AND “Albania”, “Bosnia-Herzegovina”, “Croatia”, “Greece”, “Kosovo”, “Romania” OR “Serbia”, AND “human” OR “patients”, excluding reports in animals, mosquitoes, sandflies, or ticks. In total, 43 articles were identified, showing the scarcity of available data on the presence and circulation of tick-borne encephalitis virus (TBEV), Toscana virus, West Nile virus (WNV), and Crimean–Congo haemorrhagic fever virus (CCHFV) in humans in southeastern Europe. Primarily, there are reports on TBEV in Baltic and central European countries, Toscana virus in the Mediterranean basin, and WNV and CCHFV in southern Europe. Although there is some evidence and indication of the presence of these viruses in southeast Europe, information about the extent of their circulation and burden of disease is lacking. This scarcity can be partly attributed to gaps in reporting given that not all southeastern European countries are covered by the European arbovirus surveillance system. Furthermore, available information is often heterogeneous due to the use of different case definitions and, additionally, these viruses are often not included in routine diagnostics in hospital settings.

Added value of this study

This study gives insight into the current and past circulation of TBEV, Toscana virus, WNV, and CCHFV in southeastern Europe by studying hospitalised patients presenting with arbovirus-compatible symptoms in combination with the use of multiantigen serology and a serological interpretation scheme. We showed that although data in hospital settings were often incomplete and heterogeneous regarding sample collection and timing of sample collection relative to symptom onset, patients suspected to have arbovirus infections could be differentially diagnosed using, among other tests, a multiantigen protein microarray and a diagnostic algorithm for interpretation. We found confirmed and probable recent exposures to all viruses, but primarily to Toscana virus and WNV. We also identified geographical patterns, wherein WNV was primarily found in the more northeastern countries and Toscana virus in the southwestern coastal area.

Implications of all the available evidence

The diagnostic algorithm we propose could potentially be used to strengthen routine diagnostics and improve knowledge about the circulation and burden of arboviral diseases in southeast Europe, as well as in other areas at risk for arboviral diseases. The high number of past Toscana virus exposures in this study could indicate substantial Toscana virus circulation before and during the years of sample collection, highlighting the need for the inclusion of Toscana virus in routine diagnostic testing for patients presenting to hospital in southeastern Europe with arbovirus-compatible symptoms.

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West Nile virus (WNV). Accounting for antibody kinetics, the timing of sampling (eg, early to detect IgM antibodies or approximately 2 weeks after symptom onset to detect IgG antibodies) is crucial to establish whether antibodies reflect acute infections or past exposures. Therefore, the use of IgM and IgG assays and multiplex testing for multiple related or closely related viruses is highly recommended.[5,6]

We developed a flexible, multi-antigen, antibody array platform that can be customised, allowing for the detection of circulating, clinically relevant arboviruses in specific geographical regions. Here, to assess the utility of the platform, we tested individuals presenting to hospitals with arbovirus-like symptoms in southeast Europe, a region where arboviruses are endemic, specifically focusing on four clinically relevant arboviruses: WNV, Crimean–Congo haemorrhagic fever virus (CCHFV), tick-borne encephalitis virus (TBEV), and Toscana virus.[7] The geographical range and impact of these arboviruses in southeast Europe is predicted to increase in the coming years.[8–11] By taking consecutive serum samples in the acute and convalescent phases, combined with molecular and multiplex serological testing[5,6] and confirmatory assays, we propose a classification and diagnostic scheme based on the different serological and molecular outcomes and sample timing.

Methods

Study design and patients

Here, we report an in-depth analysis of the MERMAIDS-ARBO prospective, observational study in seven countries in southeast Europe that included adults (ie, aged ≥18 years) admitted to hospital with an arbovirus-compatible syndrome, with symptom onset no more than 21 days before admission.[7] Patients were recruited from 21 hospitals located in Albania, Bosnia and Herzegovina, Croatia, Greece, Kosovo, Romania, or Serbia (appendix 2 p 5), during four arbovirus seasons (May–October) from 2016 to 2019.[12] Patients were excluded from the study if they had non-infectious CNS disorders due to hypoxic, vascular, toxic, or metabolic causes; symptoms of another confirmed cause (eg, bacterial infection, malaria, malignancy, immune disorders, or trauma); an identified focal source of infection, such as pneumonia, viral respiratory tract infection, acute infectious diarrhoea, urinary tract infection (positive urine cultures), or skin or soft-tissue infection; or symptoms caused by recurrence of a pre-existing condition. Participant-level metadata were collected with a standardised case report form (CRF) completed by local clinical staff (appendix 3 pp 1–16), which included data on demographics and vector exposure. Participant sex was self-reported (male or female) via interview on the CRFs. The protocol called for the collection of serum samples on admission (day 0) and day 7, as well as at follow-up appointments 28 days and 60 days after admission.

Full details of the selection criteria, procedures, treatments, and outcomes of the study can be found in the study protocol (appendix 4). All patients eligible for inclusion provided written informed consent. The study protocol was approved by the Oxford Tropical Research Ethics Committee (ISRCTN74074706), and all included sites gained national or local research ethics and regulatory approval, or both, as per the requirements of each country and site.

Laboratory procedures

We tested samples for arboviruses by real-time quantitative PCR (RT-qPCR) and for antibodies using a protein microarray, with confirmation by a virus neutralisation test (VNT), and ELISAs, with confirmation by an indirect immunofluorescence test (IIFT). We analysed the data of patients who had available serology and PCR data. In the linked study published separately,[7] the total number was further reduced due to incomplete data records for the clinical data analysis.

RT-qPCR

All acute-phase samples (ie, days 0 and 7) were extracted and tested individually via RT-qPCR (appendix 4 pp 16–17). For RNA purification, 100 µL of serum was used in the MagNA Pure 96 system (Roche, Basel, Switzerland) and eluted in 100 µL elution buffer with the DNA and Viral NA Small Volume Kit, according to the manufacturer's instructions. Commercial kits were used for the detection of Toscana virus RNA (TIB Molbiol, Berlin, Germany) and WNV and CCHFV RNA (Altona Diagnostics, Altona, Germany), according to the manufacturers' instructions. TBEV detection was done with an in-house assay for which primer and probe sequences were previously published.[13] For this in-house assay, we used the SuperScript III One-Step RT-qPCR System with Platinum *Taq* Polymerase (Invitrogen, Leipzig, Germany) with 12·5 µL of 2× reaction buffer provided with the kit, 0·48 µM of each primer, 0·16 µM of probe, 1 µg of bovine serum albumin, 0·8 mM $MgSO_4$ (Invitrogen), and 1 µL of enzyme mix. Thermal cycling was performed at 50°C for 15 min, followed by 95°C for 3 min and 45 cycles of 95°C for 15 s and 58°C for 20 s.

Protein microarray

Serum samples were tested for the presence of IgG and IgM antibodies specific to WNV, TBEV, or Toscana virus with a protein microarray. The protein microarray was performed as previously described, with some modifications.[5,6] In short, WNV (Sino Biological, Eschborn, Germany) and TBEV (Immune Technology, New York, NY, USA) non-structural protein 1 (NS1) and Toscana virus nucleoprotein (European Virus Archive Global, reference number 100P-01625) were printed in duplicate (two spots per antigen) on nitrocellulose glass slides. Blocker BLOTTO in tris-buffered saline (Thermo

Fisher Scientific, Waltham, MA, USA) was used to prevent non-specific antibody binding. For IgG detection, slides were incubated with 70 µL serial diluted serum samples (four-fold, 1:20 to 1:1280), followed by incubation with 70 µL secondary antibody Alexa Fluor 647-conjugated goat anti-human IgG-Fcγ (Jackson Immunoresearch, West Grove, PA, USA; research resource identifier [RRID] AB_2337889). For IgM detection, we used 70 µL serum samples first diluted 1:10 in GullSORB (Meridian Bioscience, Cincinnati, OH, USA) to remove IgG antibodies, after which Blocker BLOTTO was added to further dilute the serum to a final concentration of 1:20, followed by incubation with 70 µL Alexa Fluor 647-conjugated goat anti-human IgM-$Fc_{5\mu}$ (Jackson Immunoresearch, West Grove, PA, USA; RRID AB_2337904). For washing between incubation steps, phosphate buffered saline 0·05% TWEEN 20 washing buffer (Sigma-Aldrich, Burlington, MA, USA) was used. Antibody binding was visualised with the PowerScanner (Tecan, Männedorf, Switzerland), and the fluorescence intensity of individual spots was identified with ScanArray Express software version 4 (PerkinElmer). To obtain the 50% maximal effective concentration IgG titres, we used the median fluorescence of two identical protein spots and then R software (version 2022.12.0) to calculate titres. We used a reactive cutoff titre of at least 20 for IgG and a median fluorescence signal of at least 10 000 for IgM.

ELISA

For CCHFV, the commercial VectoCrimean-CHF-IgG and VectoCrimean-CHF-IgM ELISAs (Vector Best, Novosibirsk, Russia) were used according to the manufacturer's instructions. A test result of more than or equal to 1·00 was considered positive, within the range of 0·80–0·99 was considered equivocal, and of less than 0·80 was considered negative.

VNT

For the confirmation of WNV, TBEV, and Toscana virus reactivity on protein microarrays, we performed virus neutralisation assays. The presence of WNV lineage 2 (WNV-2)-specific and TBEV-specific neutralising antibodies was measured with an in-house VNT with some modifications.[14] In short, 100 50% tissue culture infectious dose of WNV-2 (B956; National Collection of Pathogenic Viruses, Porton Down, UK) or TBEV (Neudörfl H2J [Isolate Arb 131]) was incubated with two-fold serial diluted serum from 1:8 to 1:1024. All samples were tested in triplicate, and the geometric mean of the highest final serum dilution that completely prevented the cytopathic effect was calculated for all samples. We set the cutoff for a positive WNV-2 and TBEV VNT result at a final serum dilution above 1:16 based on an in-house validation process using WNV-2 and TBEV convalescent serum samples and serum samples from individuals who had a prior infection or vaccination with other flaviviruses (eg, yellow fever, Usutu virus, WNV, and Japanese encephalitis virus).[14]

The Toscana virus VNT was adapted from Sakhria and colleagues.[15] Briefly, 50 µL of 100 50% tissue culture infectious dose of Toscana virus strain MRS2010-4319501 (GenBank accession KC776214–KC776216) was added to two-fold serial serum dilutions from 1:10 to 1:80, except for the phosphate buffered saline controls. The serum and virus mix was incubated for 1 h at 37°C and 5% CO_2. Thereafter, a 50 µL suspension of Vero cells (Vero CCL-81; ATCC, Manassas, VA, USA), containing 2×10^5 cells in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, was added to each well and incubated at 37°C and 5% CO_2 for 5 days. The cytopathic effect was assessed by microscopy. The cutoff for a positive VNT was set at 1:40. All samples were tested in duplicate, and the geometric mean of the highest serum dilution that inhibited the cytopathic effect was calculated.

IIFT

For CCHFV, serum IgM and IgG antibodies were measured with a commercial IIFT (Mosaic 2; EUROIMMUN, Lübeck, Germany) according to the manufacturer's instructions. The cutoff for positive reactions was 1:10 for IgM and 1:100 for IgG, as recommended by EUROIMMUN.

Diagnostic algorithm

We first tested samples from day 28, when available, in the aforementioned screening assays (WNV, TBEV, and Toscana virus via protein microarray; CCHFV via ELISA). Additional participant samples (days 0, 7, and 60) were tested only from individuals with antibody binding above the cutoff at day 28 (protein microarray IgG ≥20 and IgM ≥10 000; ELISA IgG and IgM >1). When a day 28 sample was not available, the day 60 or day 7 sample was tested first, with day 60 preferred over day 7 when both were available. Day 0 was tested when all other timepoints were missing. Reactive samples on the screening assays were tested in confirmatory assays (VNT for WNV, TBEV, and Toscana virus; IIFT for CCHFV).

We estimated the number of days after symptom onset for all individuals using information provided in the CRF based on the reported day of symptom onset and the day of sampling after hospital admission. Based on the estimated days post-symptom onset, in addition to the availability of sampling timepoints (eg, four samples [complete sampling] or one, two, or three missing samples [incomplete sampling]), patients were categorised into six sampling groups to enable interpretation of the serological outcome (figure 1): (1) in the optimal sampling group, one sample was taken within 7 days of symptom onset (ie, in the acute phase) and one sample was taken at least 14 days after the first sample timepoint (ie, in the convalescent phase); (2) in the suboptimal–early group, two samples were taken within 14 days of symptom onset; (3) in the suboptimal–late group, at least two samples were taken

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See **Online** for appendix 2

See **Online** for appendix 3

See **Online** for appendix 4

Type of evidence	Type of assay	Sampling group: Optimal (one sample ≤7 DPSO, one sample ≥14 days later)	Suboptimal–early (two samples ≤14 DPSO)	Suboptimal–late (at least two samples >7 DPSO)	Non-optimal–early (at least one sample ≤7 DPSO)	Non-optimal–late (one sample >7 DPSO)	Unknown (DPSO not given or not interpretable)
Category 1: confirmed by PCR	PCR	Confirmed recent exposure	Confirmed recent exposure	Confirmed recent exposure	Confirmed recent exposure	Confirmed recent exposure	Confirmed recent exposure
Category 2: IgM, IgG, or both at any timepoint, confirmed by confirmation assay (seroconversion or ≥4-fold increase between consecutive samples)	Protein microarray plus VNT or ELISA plus IIFT	Confirmed recent exposure	Confirmed recent exposure	Confirmed recent exposure			Confirmed recent exposure
Category 3: IgM with or without IgG at any timepoint, confirmed by confirmation assay (one timepoint, multiple timepoints with no antibody kinetics or <4-fold increase)	Protein microarray plus VNT or ELISA plus IIFT	Confirmed exposed	Confirmed exposed	Confirmed exposed	Confirmed exposed	Confirmed exposed	Confirmed exposed
Category 4: IgG without IgM at any timepoint, confirmed by confirmation assay (one timepoint, multiple timepoints with no antibody kinetics or <4-fold increase)	Protein microarray plus VNT or ELISA plus IIFT	Confirmed prior exposure	Confirmed prior exposure	Confirmed prior exposure	Confirmed prior exposure	Confirmed prior exposure	Confirmed prior exposure
Category 5: IgM followed by IgG seroconversion, ≥4-fold increase in IgM or IgG, or IgM with IgG at plateau at maximum titre (multiple timepoints)	Protein microarray or ELISA	Probable exposure	Probable exposure	Probable exposure			Probable exposure
Category 6: ≥4-fold increase in IgG, no IgM	Protein microarray or ELISA	Probable exposure	Probable exposure	Probable exposure			Probable exposure
Category 7: IgM ≤7 DPSO with or without IgG (one or more timepoints), or no IgG with IgM ≤7 and <14 DPSO (multiple timepoints)	Protein microarray or ELISA	Possible exposure	Possible exposure		Possible exposure		Inconclusive
Category 8: IgG at any timepoint but no follow-up, with or without IgM (single timepoint)	Protein microarray or ELISA	Exposed	Exposed	Exposed	Exposed	Exposed	Exposed
Category 9: IgM; also IgG but no antibody kinetics (>7 DPSO; multiple timepoints)	Protein microarray or ELISA	Exposed	Exposed	Exposed			Exposed
Category 10: IgG, but no antibody kinetics (<2-fold increase or decline); no IgM (multiple timepoints)	Protein microarray or ELISA	Prior exposure	Prior exposure	Exposed			Exposed
Category 11: 2–4-fold increase in IgG, with or without IgM (multiple timepoints)	Protein microarray or ELISA	Inconclusive	Inconclusive	Inconclusive			Inconclusive
Category 12: Multiple timepoints, with negative IgG at earlier individual or multiple timepoints and positive IgG at later individual or multiple timepoints (<2-fold increase), with or without IgM	Protein microarray or ELISA	Inconclusive	Inconclusive	Inconclusive			Inconclusive
Category 13: No IgM and no IgG <14 DPSO; no follow-up	Protein microarray or ELISA	Inconclusive	Inconclusive	Inconclusive	Inconclusive	Inconclusive	Inconclusive
Category 14: IgM at one timepoint or more, no IgG (>7 DPSO); or IgM at more than one timepoint, no IgG (≤7 DPSO and ≥14 DPSO)	Protein microarray or ELISA	Inconclusive	Inconclusive	Inconclusive		Inconclusive	Inconclusive
Category 15: IgG positive and IgM negative (any timepoint), followed by IgG negative but positive IgM (>7 DPSO; multiple timepoints)	Protein microarray or ELISA	Inconclusive	Inconclusive	Inconclusive			Inconclusive
Category 16: No IgM and no IgG ≥14 DPSO (single timepoint)	Protein microarray or ELISA	Negative	Negative	Negative		Negative	Negative

Figure 1: **Diagnostic interpretation algorithm**
Per sampling group and type of evidence, nine classifications were made to interpret the laboratory results. Confirmed evidence consists of categories 1–4; the other categories comprise unconfirmed evidence. DPSO=days post-symptom onset. IIFT=indirect immunofluorescence test. VNT=virus neutralisation test.

more than 7 days after symptom onset; (4) in the non-optimal–early group, one or more samples were taken only within the first 7 days after symptom onset (ie, none in the convalescent period); (5) in the non-optimal–late group, only one sample was taken, more than 7 days post-symptom onset; and (6) in the unknown group, the number of days since symptom onset was not available in the CRF or not interpretable.

The combined consideration of the six sampling groups, molecular (RT-qPCR) results, and results of the serological assays resulted in nine possible classifications: (1) confirmed recent exposure, (2) confirmed exposed, (3) confirmed prior exposure, (4) probable exposure, (5) possible exposure, (6) exposed, (7) prior exposure, (8) inconclusive, and (9) negative (figure 1). For individuals categorised into the sampling group category of unknown, an estimation of diagnostic classification was made based on the timing of sampling after admission (day 0, day 7, day 28, or day 60), and, if available, the known days post-symptom onset of other

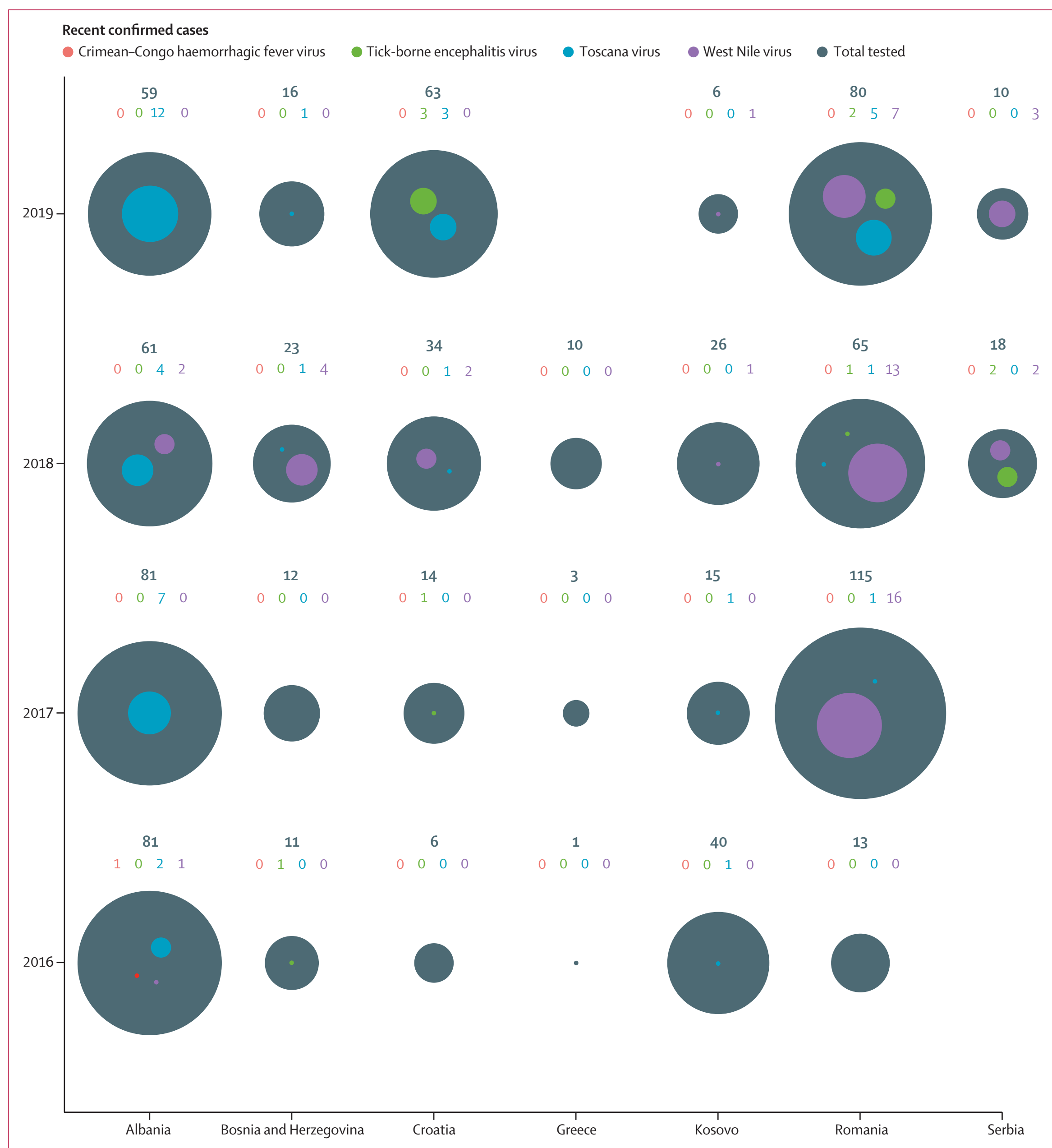


Figure 2: **Confirmed recent infections per country and sampling year**

Recent infections (diagnostic classification category 1 in figure 1) confirmed by PCR, virus neutralisation test, or indirect immunofluorescence test for each virus and the total number tested. The size of the circles corresponds to the number of samples.

available sampling timepoints, to classify the individuals into categories for type of serological evidence (figure 1).

Statistical analysis

We did a descriptive analysis by geography and year. To compare the mean age between reactive groups (evidence categories 1–10, figure 1) and not reactive groups (evidence categories 11–16, figure 1) for the studied viruses, we performed statistical *t* tests (Welch two-sample *t* test for continuous variables) using R version 4.2.2. Mean differences and odds ratios (ORs) with 95% CIs were also calculated with R version 4.2.2. For comparing categorical variables (ie, sex and insect bites) across the reactive and not reactive groups, we performed the χ^2 test when underlying test assumptions were met, followed by the pairwise proportions test (Bonferroni correction; p values were multiplied by the number of comparisons) using R version 4.2.2. When χ^2 test assumptions were violated, Fisher's exact test was used. A p value of less than 0·05 was considered significant.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between May 1 and Oct 31, during 2016–19, we screened 2896 patients for inclusion in the study. Of 913 patients eligible for inclusion after screening, 863 patients (332 women, 514 men, and 17 sex unknown) had samples sent to the study reference laboratories for molecular and serological analysis and were included in further analyses. Some individuals had insufficient clinical data to be included in the clinical analysis, but met the eligibility criteria for and were included in this serological analysis. When the timing of sampling was calculated relative to the date of symptom onset (rather than the date of admission), there was considerable variation among patients up to 40 days after symptom onset, with an average median delay of 5–6 days across all timepoints (appendix 2 p 6). This variation emphasised the need to take sample timing relative to days post-symptom onset into account when interpreting the results of the molecular and serological assays. Additionally, although 261 (30%) of 863 patients had complete sampling, 525 (61%) had samples missing from one or more timepoints, and 77 (9%) did not have data on time since symptom onset (appendix 2 p 6). Patients were most frequently sampled at hospital admission (day 0), sequentially followed by the other timepoints (appendix 2 p 6). Due to the heterogeneity in sample timing and availability, we accounted for completeness of sampling.

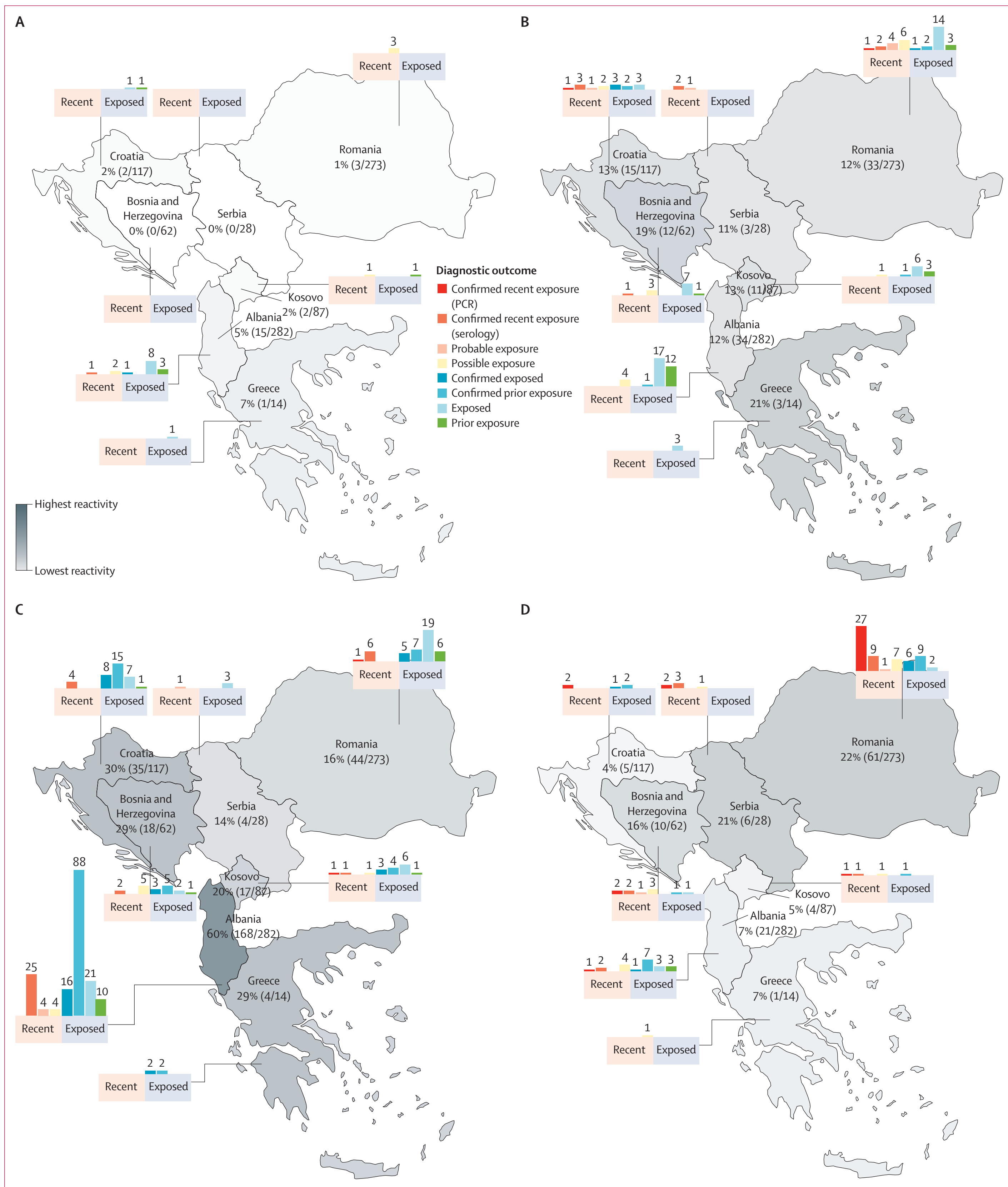
First, we categorised patients into one of the six sampling groups to be able to correctly interpret the serology results (figure 1; appendix 2 p 7). Although most included patients had incomplete or heterogeneous sampling, most (645 [75%] of 863) were categorised in the optimal (317 [37%]), suboptimal–early (128 [15%]), and suboptimal–late (200 [23%]) categories, allowing robust interpretation of test results (appendix 2 p 7). However, a notable number of patients had non-optimal serum sampling (120 [14%]) or insufficient data to allow for categorisation (ie, unknown sampling; 96 [11%]; appendix 2 p 7).

Some patients had infections confirmed by PCR, indicating a recent exposure to TBEV (two [<1%]), Toscana virus (two [<1%]), or WNV (35 [4%]; appendix 2 pp 2–4). Serologically confirmed recent infections were found most frequently for Toscana virus (38 [4%]), followed by WNV (17 [2%]) and TBEV (eight [1%]; appendix 2 pp 2–4). For Toscana virus, the recent exposures confirmed by PCR or serology were mostly seen in Albania (25 [63%] of 40), with most occurring in 2019 (12 [48%] of 25), whereas confirmed recent WNV infection was primarily detected in Romania between 2017 and 2019 (16 [14%] of 115 patients in 2017, 13 [20%] of 65 in 2018, and seven [9%] of 80 in 2019; figure 2; appendix 2 pp 2–4, 8). Only one (<1%) recent infection with CCHFV was found in Albania in 2016, confirmed by IIFT (figure 2; appendix 2 pp 2–4, 8). There were gaps in the number of inclusions in some countries or years, which was especially apparent for Greece and Serbia (figure 2).

By using the test algorithm, we separated recent exposures from prior exposures, allowing the use of the serology results to estimate background seroprevalence. Confirmed exposures that did not or probably did not occur recently were most frequently detected for Toscana virus (158 [18%] of 863), followed by WNV (28 [3%]), TBEV (ten [1%]), and CCHFV (one [<1%]; appendix 2 pp 2–4). Most patients with confirmed exposure or confirmed prior exposure to Toscana virus were also found in Albania across all 4 years (104 [66%] of 158), mostly explained by prior exposure (88 [85%] of 104; appendix 2 pp 2–4, 8).

Patients with serological evidence of infection that could not be confirmed by confirmatory assays included some patients with probable or possible recent infections: TBEV (22 [3%] of 863), WNV (19 [2%]), Toscana virus (15 [2%]), and CCHFV (six [1%]; appendix 2 pp 2–4). Patients with non-confirmed exposure or prior exposure

***Figure 3:* Overall virus reactivity and distribution of diagnostic evidence in southeast Europe**
Recent refers to all diagnostic outcomes that fall under recent exposure; exposed refers to those exposed or with prior exposure. (A) Overall Crimean–Congo haemorrhagic fever virus reactivity. (B) Overall tick-borne encephalitis virus reactivity. (C) Overall Toscana virus reactivity. (D) Overall West Nile virus reactivity. Overall virus reactivity is shown in the colours ranging from dark grey (least exposure) to light grey (most exposure) for each country. Reactive cases for each diagnostic interpretation group are shown in bar plots per country, divided into recent exposure, exposed, or prior exposure.


A
B
C
D
Diagnostic outcome
Confirmed recent exposure (PCR)
Confirmed recent exposure (serology)
Probable exposure
Possible exposure
Confirmed exposed
Confirmed prior exposure
Exposed
Prior exposure
Highest reactivity
Lowest reactivity
Recent
Exposed
Croatia 2% (2/117)
Bosnia and Herzegovina 0% (0/62)
Serbia 0% (0/28)
Romania 1% (3/273)
Kosovo 2% (2/87)
Albania 5% (15/282)
Greece 7% (1/14)
Croatia 13% (15/117)
Bosnia and Herzegovina 19% (12/62)
Serbia 11% (3/28)
Romania 12% (33/273)
Kosovo 13% (11/87)
Albania 12% (34/282)
Greece 21% (3/14)
Croatia 30% (35/117)
Bosnia and Herzegovina 29% (18/62)
Serbia 14% (4/28)
Romania 16% (44/273)
Kosovo 20% (17/87)
Albania 60% (168/282)
Greece 29% (4/14)
Croatia 4% (5/117)
Bosnia and Herzegovina 16% (10/62)
Serbia 21% (6/28)
Romania 22% (61/273)
Kosovo 5% (4/87)
Albania 7% (21/282)
Greece 7% (1/14)

were most frequently seen for Toscana virus (77 [9%]) and TBEV (69 [8%]), mostly in Albania (Toscana virus: 31 [40%]; TBEV: 29 [42%]) and Romania (Toscana virus: 25 [32%]; TBEV: 17 [25%]), and to a lesser extent for CCHFV (15 [2%]) and WNV (nine [1%]; appendix 2 pp 2–4). Around a third of patients had non-interpretable (ie, inconclusive) serological evidence for any of the four viruses (231 [27%] for Toscana virus, 264 [31%] for WNV, 265 [31%] for CCHFV, and 354 [41%] for TBEV) and, of all categories, individuals with negative test results were observed most frequently (342 [40%] for Toscana virus, 491 [57%] for WNV, 575 [67%] for CCHFV, and 398 [46%] for TBEV; appendix 2 pp 2–4).

	Not reactive	Reactive	p value	Mean difference (95% CI)	Odds ratio (95% CI)
WNV					
Total	723 (88%)	100 (12%)	..	..	..
Mean age, years (SD)	43·9 (17·7)	52·4 (18·2)	<0·0001	8·48 (–12·31 to –4·64)	..
Sex	..	..	0·34	..	..
Male	445 (62%)	56 (56%)	..	..	0·80 (0·52–1·21)
Female	278 (38%)	44 (44%)	..	..	1 (ref)
Mosquito bites	..	..	0·0002	..	..
No	616 (85%)	70 (70%)	..	..	1 (ref)
Yes	107 (15%)	30 (30%)	..	..	2·47 (1·54–3·97)
Tick bites	..	..	0·10	..	..
No	664 (92%)	97 (97%)	..	..	1 (ref)
Yes	59 (8%)	3 (3%)	..	..	0·35 (0·11–1·13)
Sandfly bites	..	..	0·43	..	..
No	710 (98%)	97 (97%)	..	..	1 (ref)
Yes	13 (2%)	3 (3%)	..	..	1·69 (0·30–6·30)
TBEV					
Total	712 (87%)	111 (13%)	..	..	..
Mean age, years (SD)	44·9 (17·9)	45·3 (18·5)	0·82	0·43 (–4·14 to 3·28)	..
Sex	..	..	0·15	..	..
Male	426 (60%)	75 (68%)	..	..	1·40 (0·91–2·14)
Female	286 (40%)	36 (32%)	..	..	1 (ref)
Mosquito bites	..	..	0·054	..	..
No	601 (84%)	85 (77%)	..	..	1 (ref)
Yes	111 (16%)	26 (23%)	..	..	1·66 (1·02–2·69)
Tick bites	..	..	0·018	..	..
No	665 (93%)	96 (86%)	..	..	1 (ref)
Yes	47 (7%)	15 (14%)	..	..	2·21 (1·19–4·11)
Sandfly bites	..	..	1·00	..	..
No	698 (98%)	109 (98%)	..	..	1 (ref)
Yes	14 (2%)	2 (2%)	..	..	0·91 (0·10–4·07)
Toscana virus					
Total	550 (67%)	273 (32%)	..	..	..
Mean age, years (SD)	42·7 (17·3)	49·6 (18·4)	<0·0001	6·97 (–9·59 to –4·35)	..
Sex	..	..	0·0055	..	..
Male	316 (57%)	185 (68%)	..	..	1·56 (1·15–2·11)
Female	234 (43%)	88 (32%)	..	..	1 (ref)
Mosquito bites	..	..	0·0002	..	..
No	439 (80%)	247 (90%)	..	..	1 (ref)
Yes	111 (20%)	26 (10%)	..	..	0·42 (0·26–0·66)
Tick bites	..	..	0·16	..	..
No	503 (91%)	258 (95%)	..	..	1 (ref)
Yes	47 (9%)	15 (5%)	..	..	0·62 (0·34–1·13)
Sandfly bites	..	..	0·33	..	..
No	537 (98%)	270 (99%)	..	..	1 (ref)
Yes	13 (2%)	3 (1%)	..	..	0·46 (0·13–1·62)

(Table continues on next page)

Overall, the most reactivity was observed towards Toscana virus (290 [34%] of 863), mainly explained by the high number of exposures and prior exposures (N=235), followed by TBEV (111 [13%]), WNV (108 [13%]), and CCHFV (23 [3%]; appendix 2 pp 2–4). Considering reactivity across all four viruses, CCHFV reactivity was low in all countries, of which Greece and Albania had the highest percentages (Greece: one [7%] of 14; Albania: 15 [5%] of 282; figure 3A, appendix 2 pp 2–4). The overall TBEV reactivity ranged from three (11%) of 28 patients in Serbia to three (21%) of 14 in Greece (figure 3B; appendix 2 pp 2–4). Toscana virus and WNV reactivity followed the same patterns as observed for recent and prior exposures; Albania had the highest overall Toscana virus reactivity (168 [60%] of 282), followed by Croatia (35 [30%] of 117), Bosnia and Herzegovina (18 [29%] of 62), and neighbouring countries Greece (four [29%] of 14) and Kosovo (17 [20%] of 87; figure 3C; appendix 2 pp 2–4). In contrast, WNV reactivity was mostly found in Romania (61 [22%] of 273), Serbia (six [21%] of 28), and Bosnia and Herzegovina (ten [16%] of 62; figure 3D; appendix 2 pp 2–4).

Patients with antibodies against WNV or Toscana virus (categories 1–10, figure 1) were significantly older than patients without (p<0·0001 for both), with substantial mean differences and narrow confidence intervals that do not include zero (table). There were no significant differences in age related to TBEV (p=0·82) or CCHFV exposure (p=0·34; table). A higher proportion of men had Toscana virus antibodies than women (OR 1·56 [95% CI 1·15–2·11]; p=0·0055; table). For WNV, TBEV, and CCHFV, no significant differences between male and female patients were observed (table).

Mosquito bites were more often reported in the WNV reactive group compared with the WNV non-reactive group, which was not the case for TBEV, Toscana virus, or CCHFV (table). Tick bites were more frequently reported for patients who were TBEV reactive versus not reactive, whereas no significant differences were seen for WNV, Toscana virus, or CCHFV reactivity (table). No significant associations were seen between reporting sandfly bites and reactivity for WNV, TBEV, Toscana virus, or CCHFV (table).

Discussion

Given the rapid worldwide spread of arboviruses, this study aimed to develop and test a diagnostic algorithm to

strengthen surveillance of clinically relevant arboviruses, including Toscana virus, WNV, TBEV, and CCHFV, in southeast Europe. We aimed to collect samples from patients presenting with arbovirus-like symptoms on days 0, 7, 28, and 60 after hospital admission, but the actual timing of sampling was heterogeneous, as is common in clinical studies. In addition, the number of follow-up samples collected decreased over time, in part because of challenges in obtaining samples from patients who had been discharged from hospital. This suboptimal sampling, which has been reported before, added an additional layer of complexity to serological diagnosis of arbovirus exposures.[16–18] However, by implementing a diagnostic algorithm that integrated the timing of sampling relative to days post-symptom onset, we were able to diagnose patients with current infections and past exposures with varying degrees of certainty.

The use of serological testing enabled the identification of both recent and prior exposures, which meant we were able to more reliably estimate virus prevalence in the included countries. Serology greatly enhanced reactivity for Toscana virus, with only two infections confirmed via RT-qPCR, whereas 38 individuals were found to be recently exposed to Toscana virus by serological VNT confirmation. The low frequency of Toscana virus infections confirmed by PCR might be explained by a very short-lived viraemia of Toscana virus in serum; however, not much is known about the kinetics of Toscana virus RNA during infection and whether they differ from other arbovirus infections.[19] The abundance of patients with Toscana virus antibodies potentially indicates substantial circulation of Toscana virus both during and before the years of sample collection and highlights the need to implement Toscana virus testing as part of standard diagnostics in clinical settings in the Balkan area, which is currently lacking.[20] However, the reasonably high number of patients who tested positive for Toscana virus antibodies might also be partly explained by co-circulation of closely related viruses, such as the sandfly fever Naples virus, that can cause cross-reactivity in serological assays.[19,21,22] This cross-reactivity was probably not an issue for the other viruses studied because of the medium to large antigenic distances between the viruses and co-circulating viruses in these areas. For WNV, although the closely related Usutu virus is prevalent in these countries, testing for Usutu virus NS1 antibodies did not show evidence for possible Usutu virus infections instead of WNV (data given in an online portal; see Data sharing).

We found geographical differences in the prevalence of acute infections and past exposures, with WNV primarily found in the more northeastern countries of the study area and Toscana virus mostly found in the southwestern coastal area. Given the known predominant circulation of Toscana virus in the Mediterranean,[21] the patterns of Toscana virus prevalence in this study seem to be in line with what has been found in the literature. We detected only a few confirmed acute TBEV and CCHFV infections, and their overall reactivity for all countries was low, suggesting a low risk of human exposure. This finding has also been described in the literature by an overall low incidence of these infections in southeast Europe.[23–25]

In the risk factor analysis, we found the odds of Toscana virus reactivity were higher in men than in women, which might be attributed to a higher occupational risk in men.[26] We did not find a significant association between Toscana virus reactivity and reporting of sandfly bites, which might be explained by the observed Toscana virus reactivity in this study being predominantly characterised by prior exposures instead of recent exposures because an association with sandfly bites would only be expected with recent exposures. We also found an association with age for WNV and TOSV, as described in previous literature.[27–30]

This study has limitations: despite implementing a diagnostic algorithm that integrated the timing of sampling relative to symptom onset, a large proportion of patients in our study had non-interpretable serological evidence (ie, inconclusive category), mainly due to heterogeneous and missing data. To aid diagnostic interpretation, sampling of patients needs to be standardised, even after they have left the hospital setting, which is challenging.[18,31] There was heterogeneity in the number of patients included from each country, with especially low numbers in Greece and Serbia given the known substantial circulation and past outbreaks of some of the target viruses in Greece and, to a lesser extent, Serbia.[21,32,33] The low numbers from these

	Not reactive	Reactive	p value	Mean difference (95% CI)	Odds ratio (95% CI)
(Continued from previous page)					
CCHFV					
Total	800 (97%)	23 (3%)	..	..	..
Mean age, years (SD)	44·8 (17·8)	49·6 (23·4)	0·34	4·78 (–14·96 to 5·40)	..
Sex	..	..	0·83	..	..
Male	486 (61%)	15 (65%)	..	..	1·21 (0·51–2·89)
Female	314 (39%)	8 (35%)	..	..	1 (ref)
Mosquito bites	..	..	1·00	..	..
No	667 (83%)	19 (83%)	..	..	1 (ref)
Yes	133 (17%)	4 (17%)	..	..	1·06 (0·26–3·25)
Tick bites	..	..	0·41	..	..
No	738 (92%)	23 (100%)	..	..	1 (ref)
Yes	62 (8%)	0	..	..	0·00 (0·00–2·13)
Sandfly bites	..	..	1·00	..	..
No	784 (98%)	23 (100%)	..	..	1 (ref)
Yes	16 (2%)	0	..	..	0·00 (0·00–9·49)

Data are n (%) unless otherwise specified. Risk factors, including age, sex, and insect bites, were tested with statistical analysis across reactive and non-reactive groups. Total number of included individuals is 823 due to missing metadata. CCHFV=Crimean–Congo haemorrhagic fever virus. TBEV=tick-borne encephalitis virus. WNV=West Nile virus.

Table: **Risk factors associated with diagnostic outcome (N=823)**

countries could be due to physicians not recognising arbovirus-compatible symptoms or local hospitals not having capacity for inclusion, sampling, or testing. This disparity also shows the limitations of our study with regards to drawing conclusions about the prevalence of the studied arboviruses in these countries. Similarly, differences in the numbers, types and sizes, and the geographical dispersion of the included hospitals in each country highlight the limitations of data representation in our study. Co-circulation of closely related viruses could have caused cross-reactivity in the serological assays, potentially resulting in overestimates, especially for unconfirmed evidence of Toscana virus. Estimates for Toscana virus could also have been affected by co-circulation of multiple Toscana virus lineages in southeast Europe—mainly lineages B and C[21]—which might have affected assay sensitivity and specificity and therefore the serological and PCR results. Most patients had no history of international travel within 21 days before illness onset, and only a few individuals reported previous arbovirus vaccination, suggesting that the possible influence from other closely related arboviruses is low.[7] Furthermore, given that most arbovirus infections are asymptomatic, and we included only adults in our study, the findings of this study probably reflect an underestimation of the overall prevalence of the studied viruses. However, by incorporating past exposures in our study design, we are able to give a reliable estimation of seroprevalence in the included regions. Another potential limitation of our study is possible recall bias of the date of symptom onset and occurrence of mosquito, tick, and sandfly bites.

In conclusion, our study shows that despite largely heterogeneous and incomplete data, setting up an efficient, targeted surveillance system with multi-antigen serology and a diagnostic algorithm is possible. The considerable presence of these viruses in southeastern Europe, notably WNV and Toscana virus, in combination with the concurrent spread of their vectors to larger geographical areas influenced by climate change, stresses the need to improve surveillance and clinical diagnostics in these countries and nearby countries that do not yet have extensive circulation of these viruses, despite the probable financial challenges.[34,35] Furthermore, this study could have implications for surveillance and research in countries considered at risk of these and other clinically important arboviruses, such as chikungunya virus, Zika virus, and dengue virus.[36–38]

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Declaration of interests

LT has received consulting fees from the UK Medicines and Healthcare products Regulatory Agency, as well as AstraZeneca and Synairgen (paid to their institution); speaker's fees from Eisai; and support for conference attendance from AstraZeneca. VBr has received speaker's fees and support for conference attendance from SC Pfizer Romania, and participated on a data and safety monitoring board for SC Pfizer Romania. VK declares having received support for conference attendance from MSD, Pfizer, and Menarini, and having participated on a data and safety monitoring board for MSD and Menarini. AMV declares having received consulting fees from MedicHub Media; having received lecture fees from Ewopharma Romania, Gilead Sciences, Zentiva, and Alfasigma Romania; having received conference attendance support from Pfizer Romania, MSD Romania, and Ewopharma Romania; having participated on a data and safety monitoring board for MSD Romania; and being President of Asociatia Medicilor Infectionisti Mures. All other authors declare no competing interests.

Data sharing

The study protocol is available as appendix 4. Fully anonymised data will be made available via an online portal (https://www.pathogensportal.nl/) after publication. The PREPARE MERMAIDS ARBO investigator group will consider requests based on validity and relevance of research questions and methods. When considered reasonable, anonymised data will be made available under data transfer agreements and coauthorship will be discussed.

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