Alimentary Pharmacology & Therapeutics

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AP&T Alimentary Pharmacology & Therapeutics

ORIGINAL ARTICLE OPEN ACCESS

Nationwide Implementation of Double Reflex Testing for Hepatitis Delta in Spain: Results From the Retrospective Phase of the Spain-DDR Study

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Received: 18 November 2025 | **Revised:** 23 December 2025 | **Accepted:** 13 January 2026

Handling Editor: Grace Wong

Keywords: anti-HDV | HDV-RNA | hepatitis delta virus | prevalence | reflex diagnostic

ABSTRACT

Background: Hepatitis delta virus accelerates liver disease progression in chronic hepatitis B, yet remains underdiagnosed. Guidelines recommend double reflex diagnostic testing with antibody testing followed by viral load testing, but uptake remains inconsistent.

Aims: To evaluate double reflex diagnostic implementation in Spain during the retrospective phase of the Spain Double Reflex Diagnostic study.

Methods: Nationwide, multicenter retrospective analysis of individuals positive for hepatitis B surface antigen (2022–2024) from 80 centers. Laboratory data included hepatitis B surface antigen, hepatitis delta virus antibody and hepatitis delta virus viral

Abbreviations: AC, autonomous communities; AEEH, Spanish association for the study of the liver; APASL, Asian Pacific association for the study of the liver; CI, confidence interval; DDR, double reflex diagnostic; EASL, European association for the study of the liver; HDV, hepatitis delta virus; IQR, interquartile ranges; LIS, laboratory information systems; SD, standard deviations.

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load. Uptake rates, hepatitis delta virus antibody positivity, viral load confirmation and viral load-confirmed prevalence were calculated with 95% confidence intervals.

Results: Among 65,763 individuals positive for hepatitis B surface antigen, hepatitis delta virus antibody testing uptake increased from 55.1% (54.4–55.8) in 2022 ($n = 18{,}986$) to 72.5% (71.9–73.1) in 2023 ($n = 22{,}463$) and 85.8% (85.1–86.4) in 2024 ($n = 24{,}314$; $p < 0.0001$). Antibody positivity remained stable (5.6%, 4.9%, 5.3%). Viral load confirmation among antibody-positive patients improved from 72.6% in 2022 to 90.8% in 2023 and 91.0% in 2024. Viral load-confirmed prevalence among antibody-positive individuals was 40.3% in 2022, 31.6% in 2023, and 30.1% in 2024. Regional heterogeneity was marked, with several autonomous communities achieving near-universal uptake (> 95%).

Conclusion: Between 2022 and 2024, double reflex diagnostic implementation expanded rapidly, demonstrating national feasibility and describing hepatitis D virus seroprevalence. However, regional inequities persist. Sustained uptake will require standardised reflex protocols, harmonization of viral load assays and continuous training to reduce underdiagnosis and improve care of coinfected patients.

1 | Introduction

Hepatitis delta virus (HDV) infection represents the most severe form of viral hepatitis, with markedly accelerated progression to cirrhosis, hepatic decompensation and hepatocellular carcinoma compared with HBV monoinfection [1–3]. Accordingly, international and regional guidelines emphasise its high morbidity and mortality and recommend systematic screening for HDV in all HBsAg-positive individuals, followed by confirmation of active infection using standardised HDV-RNA assays [4–6]. The clinical relevance of early diagnosis has further increased with the availability of effective antiviral therapies, including bulevirtide, which received full marketing authorization in the European Union in 2023 [7].

Despite these recommendations, major gaps persist in the diagnostic cascade. Current guidelines from the European Association for the Study of the Liver, the Spanish Association for the Study of the Liver, the World Health Organization and the Asian Pacific Association for the Study of the Liver uniformly recommend at least one anti-HDV test in all HBsAg-positive individuals, followed by HDV-RNA testing if serology is positive [4–6, 8, 9]. To operationalise these recommendations, several expert groups now advocate a double reflex diagnostic algorithm, in which anti-HDV testing and, if reactive, HDV-RNA testing are automatically performed from the same sample, thereby minimising diagnostic losses and delays in clinical decision-making [10].

Nevertheless, uptake of HDV testing in real-world practice remains suboptimal. In the United States, only 10.7% of 41,658 HBsAg-positive individuals underwent HDV testing within a large nationwide healthcare system [11]. In Europe, monocentric experiences have shown that automation of reflex testing increases screening coverage to nearly universal levels compared with on-demand testing [12], while Italian cohorts report that more than half of HBsAg-positive individuals remain unscreened [13]. In Spain, a nationwide cohort study among individuals with HIV/HBV coinfection found that approximately one-third had never been tested for anti-HDV [14]. In parallel, limited availability and heterogeneity of HDV-RNA assays across laboratories continue to represent a major barrier to standardised implementation of reflex-based diagnostic strategies [15].

In Spain, despite national recommendations aligned with international guidelines, systematic HDV screening has historically been inconsistently implemented. In Catalonia, introduction of an anti-HDV reflex strategy increased screening coverage from 7.6% to 93% of HBsAg-positive individuals, leading to a fivefold increase in diagnoses [16]. More recently, our group conducted a multicenter ambispective study in Andalusia, the most populated Spanish region. In its retrospective phase (2018–2022), only 18% of 18,583 HBsAg-positive individuals underwent anti-HDV testing. Following implementation of double reflex testing, screening uptake rose to 96%, HDV diagnoses increased substantially, underdiagnosis of active infection decreased from 45% to 4%, and significant diagnostic cost savings were achieved [17].

However, beyond these regional experiences, nationwide data on the real-world implementation of double reflex HDV testing across Spain are lacking. Building on prior regional studies, we established a nationwide network of 80 Clinical Microbiology Units across 16 of the 17 Spanish Autonomous Communities. In the present manuscript, we report the retrospective phase of the Spain-DDR study, aiming to quantify real-world uptake of HDV screening and double reflex testing prior to nationwide implementation, and to describe HDV seroprevalence and the proportion of RNA-confirmed infection using routinely collected laboratory data.

2 | Patients and Methods

The Spain DDR Study is a nationwide, multicenter, observational study designed to evaluate the implementation of reflex double diagnostics for HDV among HBsAg-positive individuals in Spain. The study consists of a retrospective phase (36 months, January 2022–December 2024) and a prospective phase (18 months, ongoing). The present manuscript focuses exclusively on the retrospective phase.

The study was co-coordinated by the Hospital Universitario Clínico San Cecilio of Granada and the Complexo Hospitalario Universitario of Santiago de Compostela. A total of 80 participating sites, covering 16/17 Autonomous Communities of Spain, contributed data.

Eligible participants were all adult patients (≥ 18 years) with HBsAg-positive test results recorded in the laboratory

information systems (LIS) of the participating centers during the retrospective period (2022–2024). The double reflex diagnostic algorithm was applied exclusively to HBsAg-positive samples from individuals without documented prior anti-HDV testing; samples with available previous anti-HDV results were not subjected to repeat serological or reflex testing.

Each site retrospectively extracted data from its LIS and transferred it via a standardised electronic survey (Google Forms) designed for the study. The survey captured the following information for each calendar year (2022, 2023, 2024):

1. Number of HBsAg positive patients.
2. Number of HBsAg positive patients tested for anti-HDV.
3. Number of anti-HDV positive patients
4. Number of anti-HDV positive patients tested for HDV-RNA.
5. Number of HDV-RNA positive patients

For the baseline year (2022), analyses were restricted to HBsAg-positive individuals without documented prior anti-HDV testing before the start of the study period, ensuring that uptake estimates reflected newly screened patients. For 2023 and 2024, individuals classified as not previously tested for HDV were those without any documented prior anti-HDV testing at any time point recorded in the laboratory information systems. As part of the predefined laboratory information system search criteria, individuals were deduplicated within each calendar year, and repeated records of the same individual across consecutive years were excluded, ensuring that each individual was counted only once and minimizing the risk of denominator inflation due to repeated testing.

Centers also reported whether reflex testing protocols (anti-HDV or HDV-RNA automatically triggered from the same sample) were implemented, whether HDV-RNA testing was available on-site or required referral to an external reference laboratory, and the laboratory assays used for anti-HDV and HDV-RNA testing.

The primary endpoint was the proportion of HBsAg-positive patients tested for anti-HDV antibodies during the retrospective period. Secondary endpoints included: (a) Proportion of anti-HDV-positive patients tested for HDV-RNA; (b) Proportion of confirmed active HDV infection (HDV-RNA positivity); (c) Regional distribution of testing uptake and prevalence of anti-HDV and HDV-RNA positivity across Spain; (d) Identification of laboratory-related barriers, including absence of reflex testing and lack of HDV-RNA availability.

2.1 | Statistical Analysis

Categorical variables were summarised as frequencies and percentages. Continuous variables were expressed as means with standard deviations (SD) or medians with interquartile ranges (IQR), as appropriate. Comparisons between groups were performed using chi-square or Fisher's exact tests for categorical variables, and Student's *t*-test or Mann–Whitney *U* test for continuous variables. Regional variation in uptake and prevalence was described. A two-sided p-value < 0.05 was considered statistically significant. Missing data were handled by complete-case analysis, with sensitivity analyses using imputation where appropriate. Statistics were performed using R Studio and Graph Pad Prism V8 software.

3 | Results

3.1 | Diagnostic Cascade Across Spain, 2022–2024

During 2022, across 18,986 HBsAg-positive individuals from 69 centers in 16 Autonomous Communities (ACs), 10,465 underwent anti-HDV testing: 55.1% (95% CI 54.4–55.8). Of those tested, 581 were anti-HDV positive, 5.6% (95% CI 5.1–6.0). HDV-RNA testing was available for 422/581 anti-HDV-positive patients, 72.6% (95% CI 68.8–76.2), of whom 170 were HDV-RNA positive, 40.3% (95% CI 35.6–44.9). By 2023, a total of 22,463 HBsAg-positive individuals, again from 69 centers in 16 ACs, were recorded. Among the 20,089 not previously tested for HDV, 14,565 underwent anti-HDV testing: 72.5% (95% CI 71.9–73.1). Of those tested, 715 were anti-HDV positive, 4.9% (95% CI 4.6–5.3). Confirmatory RNA data were available for 649/715, 90.8% (95% CI 88.4–92.8); 205 were HDV-RNA positive, making an RNA-confirmed prevalence of 31.6% (95% CI 28.0–35.3). Compared with 2022, anti-HDV uptake increased significantly (55.1% to 72.5%, $p < 0.0001$), accompanied by improved RNA confirmation (72.6% to 90.8%, $p < 0.0001$). In 2024, we identified 24,314 HBsAg-positive individuals from 80 centers in 16 ACs. Among the 12,227 not previously tested for HDV, 10,487 were tested in 2024: 85.8% (95% CI 85.1–86.4). Among those tested, 559 were anti-HDV positive, 5.3% (95% CI 4.9–5.8). RNA data were available for 509/559, 91.0% (95% CI 88.4–93.3); 158/509 were HDV-RNA positive, 31.0% prevalence (95% CI 27.0–35.3). Compared with 2023, the uptake of anti-HDV testing increased significantly (72.5% vs. 85.8%; $p < 0.0001$). These results are summarised in Table 1, which provides an overview of the HDV diagnostic cascade across Spain from 2022 to 2024, including testing uptake and positivity rates.

3.2 | Laboratory Methods Used for Anti-HDV and HDV-RNA Testing

Anti-HDV testing across participating centers was primarily performed using the LIAISON XL MUREX Anti-HDV CLIA (DiaSorin), accounting for approximately 72% of centers, followed by HDV Ab Total Antibody ELISA (Dia. Pro Diagnostics) in 22% and combined HDV IgG/HDV IgM ELISAs (Dia. Pro Diagnostics) in 6%. HDV-RNA detection was most commonly carried out using the Hepatitis Delta Real-Time PCR Kit (Vircell) in 89% of centers (limit of detection [LoD] 23 IU/mL), followed by the LightMix Kit Hepatitis D Virus (HDV) (Roche/TIB Molbiol) in 6% (LoD 35–80 IU/mL, estimated from manufacturer specifications), and the RealStar HDV RT-PCR Kit 1.0 (altona Diagnostics) in 5% (LoD 10–15 IU/mL). These data reflect the heterogeneity of diagnostic platforms used across centers for both serological screening and RNA confirmation.

3.3 | Temporal Trends

Anti-HDV uptake rose significantly from 55.1% in 2022 to 72.5% in 2023 ($p < 0.0001$), with this increase occurring in

TABLE 1 | Diagnostic cascade by year (2022–2024).

Year	HBsAg+ (*N*)	Anti-HDV tested % (95% CI[a])	Anti-HDV+ *N*, (%) [95% CI]	RNA tested % (95% CI)	RNA+ *N*, (%) [95% CI]
2022	18,986	55.1 (54.4–55.8)	581 (5.6) [5.1–6.0]	72.6 (68.8–76.2)	170 (40.3) [35.6–44.9]
2023	22,463	72.5 (71.9–73.1)	715 (4.9) [4.6–5.3]	90.8 (88.4–92.8)	205 (31.6) [28.0–35.3]
2024	24,314	85.8 (85.1–86.4)	559 (5.3) [4.9–5.8]	91.0 (88.4–93.3)	158 (31.0) [27.0–35.3]

Note: Summary of HDV diagnostic cascade across Spain, showing the number of HBsAg-positive individuals, uptake of anti-HDV reflex testing, number and proportion of anti-HDV-positive cases, uptake of confirmatory HDV-RNA testing, number and proportion of RNA-positive cases, and overall RNA-confirmed prevalence among all HBsAg-positive individuals. Data are presented as percentages with 95% CIs.
[a]Confidence Interval.

temporal association with the initiation of the national project and multiple dissemination activities, including presentations at national and international conferences, dissemination of DDR-Andalucía results and local meetings with leaders from each Autonomous Community. Uptake further increased to 85.8% in 2024 ($p < 0.0001$ vs. 2023), demonstrating the scalability of DDR implementation across Spain. Anti-HDV positivity rates remained stable across the study period: 5.6% in 2022, 4.9% in 2023 and 5.3% in 2024. RNA confirmation among anti-HDV-positive patients improved markedly between 2022 (72.6%) and 2023 (90.8%, $p < 0.0001$), and remained high in 2024 (91.0%), indicating sustained improvements in confirmatory testing. The RNA-confirmed prevalence of HDV infection among anti-HDV positive individuals was 40.3% in 2022, 31.6% in 2023 and 30.1% in 2024 ($p = 0.0015$, 2022 vs. 2023; $p < 0.0012022$ vs. 2024).

3.4 | Uptake of DDR by Autonomous Communities

Marked heterogeneity was observed across Spain in the implementation of anti-HDV reflex testing and RNA confirmation between 2022 and 2024. Andalusia, Canary Islands and Galicia rapidly achieved >90% anti-HDV uptake, sustaining near-universal testing (>95%) in 2024, with RNA confirmation rates >90%. Valencia, Madrid and Basque Country also reached >95% uptake by 2024, although RNA confirmation remained slightly lower in Madrid (79%) and Basque Country (84%). Castile and León, La Rioja, and Extremadura started from very low uptake in 2022 (15%–20%) but reached near 100% uptake in 2024, with excellent RNA confirmation rates. Murcia and Cantabria showed steady increases, exceeding 70%–80% uptake by 2024 with RNA confirmation >90%. Castilla–La Mancha, Aragón, and Navarre displayed irregular trajectories, with improvements in some years followed by declines. Overall, by 2024, 11 of 16 ACs reported >70% uptake, and in at least 8 regions, uptake was >95%, demonstrating the feasibility of nationwide implementation but also the persistence of regional inequities. Detailed results are presented in Table 2, while the temporal evolution of anti-HDV and RNA uptake across ACs is depicted in Figures 1 and 2.

4 | Discussion

In this nationwide, multicenter study including nearly 66,000 HBsAg-positive individuals across Spain between 2022 and 2024, we observed a substantial increase in the uptake of HDV testing over time, but significant heterogeneity across regions remains. Anti-HDV testing coverage rose markedly from 55.1% in 2022 to 72.5% in 2023, and further to 85.8% in 2024, indicating the feasibility and scalability of double reflex (DDR) implementation in diverse healthcare settings. However, these findings should be interpreted in light of the retrospective design of the study and the reliance on aggregated laboratory information system data, which limits causal inference and detailed clinical interpretation.

These results contrast with previous reports from other countries, where uptake of HDV testing remains very limited. In the United States, fewer than 11% of HBsAg-positive patients have ever been tested for HDV [11]. In Italy, more than half of HBsAg-positive individuals remain unscreened [13], and uptake varies even where reflex testing has been introduced [12]. Similar limitations have been highlighted in Germany, where retrospective analyses revealed that only 20% of HBsAg-positive cases were screened for anti-HDV in routine practice, although a prospective pilot in primary care demonstrated the feasibility of DDR implementation [18]. Likewise, a study from Austria showed that reflex testing in tertiary care substantially increased diagnostic yield compared to standard on-demand testing [12]. Importantly, community-based data from Pakistan illustrate the benefits of DDR in high-prevalence settings, revealing very high rates of anti-HDV positivity (67%) and active viremia (58%) among HBsAg carriers [19]. At the global level, the Polaris Observatory has emphasised that DDR represents the most efficient strategy to generate accurate prevalence estimates and identify undiagnosed patients, even in countries with low HBV prevalence [10]. Our findings highlight that Spain has made rapid progress in scaling DDR testing, with over two-thirds of regions achieving >70% uptake and several Autonomous Communities reaching >95% by 2024. Nonetheless, some areas showed fluctuating or persistently low uptake, illustrating that full nationwide implementation remains challenging.

Importantly, the interpretation of regional differences requires caution, as Autonomous Communities are not directly comparable in terms of population size, laboratory organization, diagnostic capacity and workflow structures. The observed heterogeneity in DDR uptake and RNA confirmation rates may be partially explained by structural and organisational factors, such as the degree of laboratory centralization, the availability of in-house HDV-RNA testing versus referral-based workflows, pre-existing reflex testing policies and the presence of local clinical or laboratory leadership driving implementation. Differences in the pace of adoption across regions may also reflect variations in

TABLE 2 | Uptake of double reflex HDV testing by Autonomous Communities in Spain (2022–2024).

Autonomous community	2022 Anti-HDV tested % (95% CI[a])	2022 RNA tested % (95% CI)	2023 Anti-HDV tested % (95% CI)	2023 RNA tested % (95% CI)	2024 Anti-HDV tested % (95% CI)	2024 RNA tested % (95% CI)
Asturias	60.6 (55.9–65.2)	50.0 (15.7–84.3)	63.1 (58.5–67.6)	100 (79.4–100)	100 (97.3–100)	100 (15.8–100)
Extremadura	15.2 (11.5–19.5)	0 (NA)	65.5 (61.5–69.4)	18.2 (0–91.8)	100 (97.2–100)	100 (39.8–100)
La Rioja	20.1 (15.0–26.0)	0 (NA)	54.7 (46.3–62.9)	0 (NA)	100 (98.5–100)	100 (63.1–100)
Valencia	73.4 (70.1–76.5)	100 (92.5–100)	78.1 (75.3–80.7)	83.6 (70.6–92.5)	100 (99.7–100)	100 (94.1–100)
Galicia	56.6 (53.7–59.5)	95.0 (74.4–99.9)	100 (99.6–100)	90.5 (76.5–97.6)	99.4 (98.5–99.8)	100 (78.2–100)
Madrid	51.0 (49.1–52.9)	63.2 (51.2–74.1)	68.0 (66.2–69.7)	89.8 (82.0–95.0)	99.4 (99.1–99.7)	79.2 (68.4–87.7)
Basque Country	63.5 (61.5–65.4)	48.5 (33.9–63.2)	70.1 (68.3–71.9)	93.9 (86.9–97.8)	98.1 (97.5–98.6)	84.0 (69.4–93.5)
Andalusia	59.8 (58.3–61.2)	80.0 (71.6–86.8)	93.1 (92.5–93.7)	88.9 (82.9–93.3)	95.4 (94.9–95.9)	92.7 (87.5–96.2)
Canary Islands	98.7 (98.0–99.2)	66.7 (50.1–80.8)	73.4 (71.1–75.6)	81.2 (70.0–89.6)	93.2 (91.9–94.3)	100 (87.7–100)
Cantabria	65.0 (59.8–69.9)	28.6 (1.1–82.8)	77.1 (72.4–81.3)	46.2 (9.8–86.1)	82.5 (79.2–85.5)	100 (54.1–100)
Navarre	64.8 (60.9–68.6)	93.3 (66.7–99.9)	68.6 (64.8–72.2)	87.5 (59.3–98.7)	74.5 (71.0–77.8)	100 (63.1–100)
Murcia	45.6 (41.6–49.6)	57.1 (26.7–84.0)	60.1 (56.4–63.7)	61.5 (34.5–84.1)	73.2 (70.6–75.7)	92.9 (75.7–99.2)
Castile and León	15.8 (13.9–17.8)	97.0 (80.1–99.9)	25.0 (22.8–27.4)	100 (94.8–100)	68.8 (66.6–71.0)	96.2 (79.9–99.9)
Balearic Islands	48.6 (44.7–52.7)	12.5 (0–79.8)	26.2 (22.4–30.3)	0 (NA)	66.4 (62.1–70.5)	87.5 (44.0–99.8)
Aragón	4.2 (2.2–7.2)	100 (15.8–100)	53.0 (47.3–58.6)	92.9 (64.8–99.9)	47.8 (42.3–53.4)	100 (71.5–100)
Castilla–La Mancha	59.7 (56.7–62.6)	45.0 (14.1–79.2)	68.4 (65.4–71.3)	21.7 (0.7–72.9)	41.4 (38.9–43.9)	72.0 (46.3–90.2)

Note: Proportion of HBsAg-positive individuals undergoing reflex anti-HDV testing and confirmatory HDV-RNA testing across 16 Autonomous Communities. Data are shown as percentages with 95% confidence intervals (CIs). Results are presented by year to facilitate temporal and regional comparison. Marked heterogeneity was observed across regions and years, reflecting differences in the pace and extent of implementation rather than underlying epidemiological variation. Some Autonomous Communities (e.g., Andalusia, Galicia, Canary Islands) rapidly achieved high or near-universal uptake (> 90% by 2024), whereas others (e.g., Aragón, Castilla–La Mancha) showed delayed, fluctuating, or incomplete implementation. These differences should be interpreted in the context of regional variability in laboratory organization, testing availability, and workflow structures.
[a]Confidence interval.

resource allocation and coordination between microbiology and hepatology services. Rather than indicating intrinsic differences in disease burden, these inequities are therefore more likely to reflect differences in healthcare organization and diagnostic implementation.

The proportion of anti-HDV positivity remained stable across the 3 years (5%–6%), consistent with previously reported prevalence in Mediterranean cohorts [3, 16, 17, 20, 21]. In contrast, the prevalence of RNA-confirmed infection among anti-HDV–positive individuals decreased from 40.3% in 2022 to 31.6% in 2023 ($p = 0.0018$) and 30.1% in 2024 ($p = 0.00055$), with no significant difference between 2023 and 2024 ($p = 0.62$). This proportion is lower than that reported in meta-analyses and tertiary European series (≈58%) [3, 5], which may in part reflect differences in study design and testing strategies and likely because the progressive implementation of a double-reflex diagnostic (DDR) algorithm shifted testing from patients with high clinical suspicion to near-universal reflex screening of HBsAg-positive individuals, thereby capturing a larger number of past infections or low-replicative disease and yielding more representative estimates of active infection. Consistent with this interpretation, cohorts using reflex testing [16, 17, 22] report similar proportions (40%–45%), whereas hepatology-based series in Spain still report values > 60% [16, 23]. In addition, the heterogeneity of diagnostic platforms used across centers, including differences in analytical sensitivity, limits of detection and assay standardisation for HDV-RNA, may have influenced confirmation rates and contributed to the observed temporal decline in RNA positivity. Therefore, this trend should be interpreted with caution and should not be assumed to reflect a true epidemiological change.

Our results extend the findings of the Andalusia DDR study, which showed that reflex testing was feasible, increased diagnosis, minimised under-diagnosis and was cost-saving [17]. Similar benefits were reported by Palom et al. in a single-center study in Catalonia, where implementation of anti-HDV reflex testing among HBsAg-positive individuals substantially increased testing rates [16]. At the national level, the increases in testing uptake observed in 2023 occurred in temporal association with the initiation of the Spain DDR project, national and international conference presentations, dissemination of

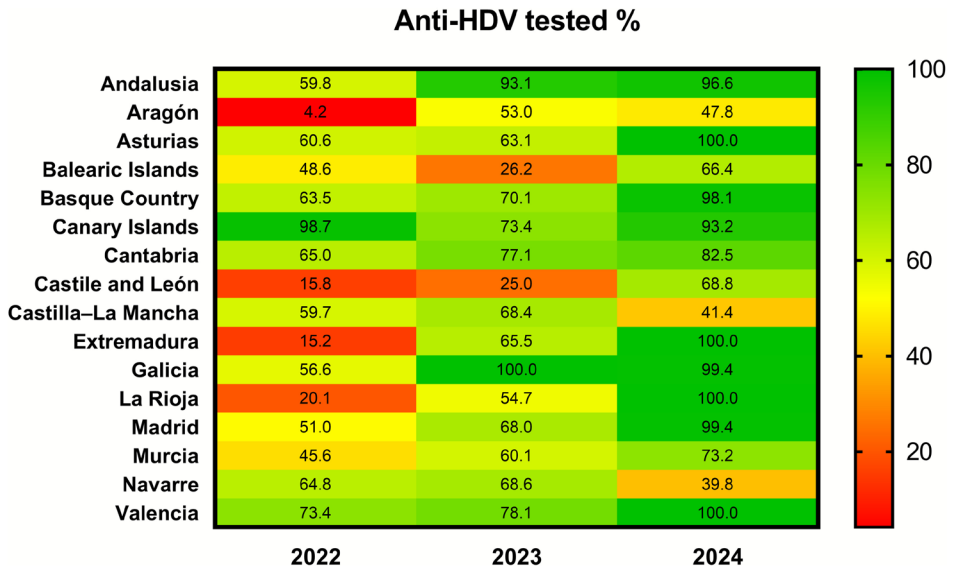

FIGURE 1 | Uptake of reflex anti-HDV testing by Autonomous Communities in Spain (2022–2024). Heatmap showing the proportion of HBsAg-positive individuals undergoing reflex anti-HDV testing across 16 Autonomous Communities in 2022, 2023, and 2024. Values are presented as percentages, with a colour gradient from red (lowest uptake) to green (highest uptake). Autonomous Communities are displayed consistently across years to allow direct visual comparison of temporal implementation patterns. Marked heterogeneity was observed across regions and years. Several Autonomous Communities (e.g., Andalusia, Galicia, Canary Islands, and Valencia) showed rapid progression toward high or near-universal uptake by 2024, whereas others (e.g., Aragón, Castilla–La Mancha, and Navarre) exhibited delayed, fluctuating, or incomplete implementation. These patterns highlight regional inequities in the pace of diagnostic rollout rather than differences in disease burden.

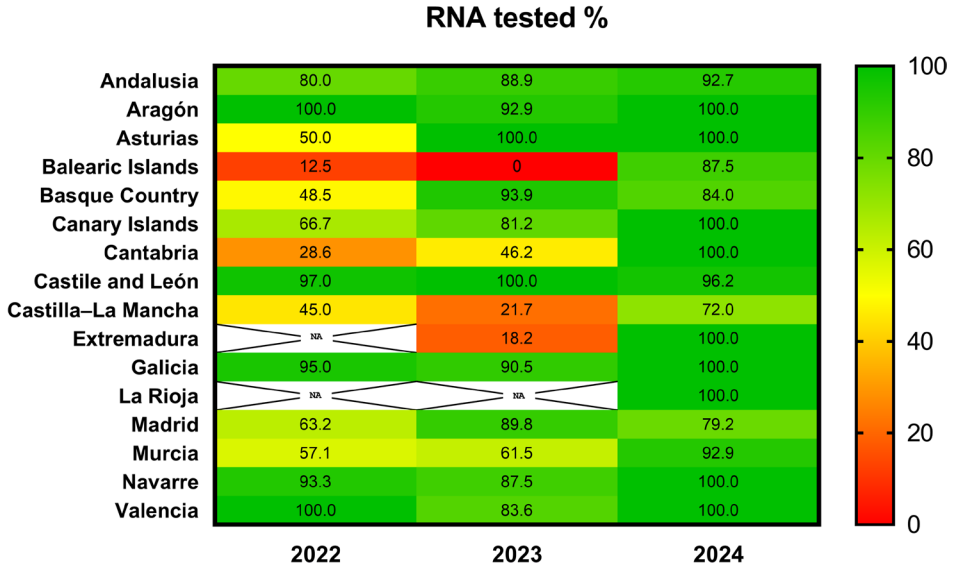

FIGURE 2 | Uptake of confirmatory HDV-RNA testing by Autonomous Communities in Spain (2022–2024). Heatmap showing the proportion of anti-HDV–positive individuals who underwent confirmatory HDV-RNA testing across 16 Autonomous Communities in 2022, 2023, and 2024. Values are presented as percentages, with a colour gradient from red (lowest uptake) to green (highest uptake). Autonomous Communities are displayed consistently across years to facilitate direct visual comparison of confirmatory testing practices over time. Cells marked as "NA" indicate Autonomous Communities where no anti-HDV–positive cases were identified in a given year or where HDV-RNA testing was not available, and therefore RNA confirmation could not be assessed. The heatmap highlights substantial regional and temporal variability in confirmatory testing workflows, with progressive improvement in most regions and near-universal RNA confirmation achieved by 2024 in the majority of Autonomous Communities. Residual disparities observed in earlier years and in selected regions are likely to reflect differences in testing availability, laboratory organization, and referral pathways rather than true differences in HDV epidemiology.

the Andalusian experience and regional leadership meetings across Autonomous Communities. The continued improvements observed in 2024 demonstrate that DDR can be scaled up nationally, but regional inequities persist and require tailored interventions. Sustained implementation will depend on centralised quality control, harmonisation of HDV-RNA assays, continuous training and full integration of reflex testing into routine laboratory workflows.

Several limitations of this retrospective analysis should be acknowledged. First, the study relied on laboratory information systems, which may not capture all relevant clinical variables or reasons for missing tests. In addition, the laboratory-based nature of the dataset did not allow differentiation between newly diagnosed HBsAg-positive individuals and patients already in follow-up. Second, the aggregated nature of the dataset limits the ability to draw epidemiological or clinical inferences beyond descriptive proportions. Third, differences in assay availability, analytical performance, local diagnostic definitions and regional workflows may have influenced uptake and confirmation rates, as well as the interpretation of temporal trends and interregional comparability. Fourth, although our network covered approximately two-thirds of the Spanish population, practices in nonparticipating centers may differ. In addition, only 16 of the 17 Spanish Autonomous Communities were included, as Catalonia had already been the subject of a dedicated study evaluating the implementation of anti-HDV reflex testing among HBsAg-positive individuals. Nevertheless, this is by far the largest study of HDV testing practices in Spain and one of the largest worldwide.

In conclusion, the retrospective phase of the Spain DDR study shows that, despite clear guideline recommendations, the implementation of HDV reflex testing in Spain remains heterogeneous, with persistent regional inequities. The improvements observed between 2022 and 2024 indicate an increased uptake of DDR at the national level and support the feasibility of broader implementation. In line with recent international reports, our findings support the consideration of DDR as a standard strategy for HDV diagnosis to facilitate patient management and improve the quality of epidemiological surveillance data. Future efforts should focus on sustaining uptake through integration into national laboratory protocols, harmonization of HDV-RNA testing and ongoing educational initiatives. The forthcoming prospective and molecular epidemiology phases of the Spain DDR project will allow a more detailed assessment of the clinical and public health implications of systematic HDV testing.

Author Contributions

A.A., A.d.S. and F.G. conceived and designed the study. All authors participated in data collection. A.A., A.d.S. and F.G. performed the data analysis and contributed to data interpretation. A.A., A.d.S., A.A. and F.G. drafted the manuscript. All authors participated in revising the article critically for important intellectual content and approved the final submitted version.

Funding

This work was supported by Gilead Sciences, IN-ES-980-7036.

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Acknowledgements

Financial support was received from Gilead through the Spearhead program (IN-ES-980-7036). A. d. S. is supported by 'Instituto de Salud Carlos III' (grant number JR22/00055). During the preparation of this work, the authors used ChatGPT (OpenAI, San Francisco, CA, USA) to perform stylistic revision of paragraphs written by the authors; it did not generate new content. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication. All authors approved the final version of the article, including the authorship list. Federico García is the guarantor of this article.

Ethics Statement

The study was conducted in accordance with the principles of the Declaration of Helsinki and Spanish regulations on biomedical research and personal data protection. The protocol was approved by the Andalusian Biomedical Research Ethics Committee.

Consent

The retrospective phase used anonymized laboratory data only; therefore, informed consent was waived.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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