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New Transport Medium for Cultural Recovery of *Helicobacter pylori*

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We developed a new transport medium (GESA—*Helicobacter pylori* transport medium [publication no. WO/2014/019696, patent pending no. PCT/EP2013/002292; Liofilchem s.r.l., Roseto degli Abruzzi, Teramo, Italy]) for recovery of *Helicobacter pylori* from gastric biopsy samples. GESA transport medium, in a semisolid state, provides the optimal conditions for maintaining the viability of the microorganism over time. The efficacy of the transport medium was assessed through *in vitro* and *ex vivo* experiments. We were able to recover different suspensions of *H. pylori* ATCC 43629 and *H. pylori* 13 A in GESA transport medium stored at 4°C for up to 10 days. In particular, with a starting inoculum of ∼10^5 CFU, after 7 days of storage, 150 ± 25 CFU and 40 ± 7 CFU of the reference and clinical strains were detected, respectively. *H. pylori* colonies were isolated from gastric specimens taken from both the antrum and the fundus in 68 (90.66%) of 75 urea breath test (UBT)-positive patients. Moreover, GESA transport medium allowed the recovery and isolation of *H. pylori* colonies from additional biopsy samples from 13 of the 75 detected subjects at up to 10 days of biopsy sample storage at 4°C. Finally, GESA transport medium preserved its characteristics when stored at 4°C for 1 year from its preparation, thus allowing good recovery of *H. pylori*. GESA transport medium can be considered a standardized transport medium with high performance that optimizes the recovery rate of *H. pylori* grown by culture.

Helicobacter pylori, the principal species of the genus *Helicobacter*, colonizes the human stomach early in the life of the host and tends to persist over time (1). The microorganism is also responsible for a variety of gastroduodenal pathologies, and it was also described as a risk factor for gastric carcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (2, 3).

Culture of *H. pylori* from gastric biopsy specimens represents a more accurate method for diagnosis in patients with suspected infection, with 100% specificity and a level of sensitivity which depends on the performing conditions (4–6). Currently recommended for populations with a high prevalence of drug resistance, culture allows isolation of the microorganism for testing susceptibility to antimicrobials before starting appropriate treatment (7, 8). Moreover, the isolation of the microorganism allows analysis of the genotypic status of the isolates, offering important information for bacterial characterization and, consequently, for therapy evaluation (9, 10).

Culture of *H. pylori*, however, can prove difficult because of the fastidious nature of the microorganism and its capability to become unculturable (11–14). Multiple gastric biopsy specimens and suitable transport media represent important tools for a high diagnostic yield (15, 16).

Therefore, to make *H. pylori* culture from gastric biopsy specimens possible in an endoscopy unit, rapid and efficient transportation to microbiological laboratories is essential, taking into account that the survival rate of *H. pylori* can be affected by both the time lapse between sampling and culture and the transport temperature (17).

Previous studies on the optimal transport conditions for *H. pylori* were conducted to minimize the loss of bacterial cultivability, increasing the recovery of the colonies (17–21).

In this report, we propose a new semisolid transport medium for cultural recovery and isolation of *H. pylori* for subsequent drug susceptibility testing from biopsy specimens, adding useful hints to enhance the recovery rate and to preserve the viability of the strains during long-term transportation.

MATERIALS AND METHODS

Composition of GESA transport medium. GESA transport medium (Liofilchem s.r.l., Roseto degli Abruzzi, Teramo, Italy) for storage and transport of gastric specimens for the detection of *H. pylori* is based on the combination of soft Granulated agar (Liofilchem) (7 g/liter) with 10 g/liter of enzymatic digest of casein (Liofilchem), 9.5 g/liter of enzymatic digest of animal tissue (Liofilchem), 2 g/liter of yeast extract (Liofilchem), 1 g/liter of glucose (Liofilchem), and 5.5 g/liter of sodium chloride (Liofilchem) at pH 7.0 ± 0.2 in distilled water. After sterilization, 10 ml of enrichment products is added to the mixture (Table 1).

The efficiency of the semisolid transport medium in viable bacterial preservation was tested through the use of inocula of both different bacterial suspensions and biopsy specimens.

The performance of the transport medium, stored at 4°C, was evaluated until 12 months after its preparation.

***H. pylori* suspensions and viability assay.** For quantitative assessment of recovery of bacteria from GESA transport medium, serial dilutions of the reference strain *H. pylori* ATCC 43629 (ATCC LGC Standards S.r.l., Milan, Italy) and the clinical strain *H. pylori* 13 A were used. For the experiments, the strains were grown in chocolate agar plus 1% IsoVitaleX (AC; Becton Dickinson Italia, Milan, Italy) at 37°C for 5 days under microaerobic conditions consisting of 85% N_2, 5% O_2, and 10% CO_2 (Rivoira, Milan, Italy). From each strain, a bacterial suspension in brucella broth (BB; Biolife, Milan, Italy) was prepared to achieve an optical density at 600 nm (OD_{600}) of ∼0.2, corresponding to approximately 3.5×10^7 CFU/ml, and serial 10-fold dilutions were prepared at up to 10^{-5} times the original broth culture concentration. Ten-microliter volumes of each dilution were inoculated in duplicate on 150 μl of GESA transport medium and stored at 4°C until the 10-day time point. In addition, 10 μl of the first

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TABLE 1 Composition of GESA transport medium

Ingredient(s) (unit) or parameter	Value(s)
Enrichment products (ml)[a]	10
Granulated agar (g)	7
Enzymatic digest of casein (g)	10
Enzymatic digest of animal tissue (g)	9.5
Yeast extract (g)	2
Glucose (g)	1
Sodium chloride (g)	5.5
Distilled H_2O (ml)	961.2
pH	7.0 ± 0.2

[a] Vitamin B12, 0.01 g/liter; L-glutamine, 10.0 g/liter; adenine, 1.0 g/liter; guanine hydrochloride, 0.03 g/liter; *p*-aminobenzoic acid, 0.013 g/liter; nicotinamide adenine dinucleotide, 0.25 g/liter; thiamine pyrophosphate, 0.1 g/liter; ferric nitrate, 0.02 g/liter; thiamine hydrochloride, 0.003 g/liter; L-cysteine hydrochloride, 25.9 g/liter; L-cystine, 1.1 g/liter; dextrose, 100.0 g/liter.

dilution was inoculated on GESA transport medium under the same conditions for the detection of the morphology and viability of the bacteria.

For the bacterial recovery assessments, at time zero and after 1, 2, 3, 4, 5, 7, and 10 days, the semisolid cylinders of GESA transport medium containing the bacterial suspensions were collected, spread on AC, and incubated for 3 to 5 days under microaerobic conditions. At each time point and for each broth culture dilution, the number of CFU was counted. Three experiments were performed in duplicate, and the CFU detected were indicated as the mean of the numbers of colonies counted independently by two microbiologists.

Similarly, for the viability test, Live/Dead Backlight bacterial viability staining (Molecular Probes Inc./Invitrogen, Italy) was performed directly on the inoculated semisolid cylinders of GESA transport medium; the morphology and viability of bacteria were visualized under a fluorescent Leika 4000 DM microscope (Germany). As for the bacterial shape, the number of spiral/bacillary (B) and coccoid (C) forms was determined by counting 10 randomly chosen fields of view. Two microbiologists carried out counts independently. The detected U-shaped bacteria were considered coccoid cells.

Biopsy specimens. For the evaluation of the reliability of GESA transport medium in recovering *H. pylori* by gastric biopsies, 75 patients with a positive result from a ^{13}C urea breath test (UBT), performed with citric acid and 75 mg of ^{13}C urea, were included in this study. Gastric biopsy specimens were collected from endoscopy units in the Abruzzo area, in Italy. Two biopsy specimens both from the gastric antrum and from the fundus were taken from each subject for a total of 150 analyzed specimens, and from 13 of them, 6 additional biopsy samples were collected. Patients gave their informed consent to the study.

After endoscopy, biopsy specimens from the antrum and fundus were immediately put in GESA transport medium and processed within 2 h (day 1). The additional samples were stored in GESA transport medium at 4°C and randomly processed for up to 10 days.

Biopsy specimens were trimmed with a razor, homogenized, and cultured on chocolate agar plus 1% IsoVitaleX (CA; Becton Dickinson & Co., Cockeysville, MD) and Campylobacter selective medium (CP; Oxoid). Plates were incubated under microaerobic conditions at 37°C for 3 to 5 days.

H. pylori colonies were identified on the basis of their colony morphology, Gram staining, and positive reaction with urease, catalase, and oxidase.

Statistical analysis. The statistical significance of the differences between the results determined for the experimental groups was evaluated using the Student *t* test. Probability levels of <0.05 were considered statistically significant.

RESULTS

Recovery of *H. pylori* from suspensions. Table 2 displays the recovery rate of *H. pylori* suspensions after storage in GESA trans-

TABLE 2 Number of CFU per plate obtained from *Helicobacter pylori*[a]

Dilution of starting bacterial suspensions (10 μl) (OD_{600}, ca. 0.2)	No. of CFU per plate at the indicated no. of days of storage at 4°C															
	0		1		2		3		4		5		7		10	
	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C
10	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	324 ± 35	150 ± 25	40 ± 7	4 ± 3	3
10^{-1}	$>10^3$	$>10^3$	$>10^3$	705 ± 90	$>10^3$	208 ± 30	$>10^3$	155 ± 25	321 ± 35	98 ± 20	375 ± 40	8 ± 3	3	0	2	1
10^{-2}	490 ± 70	106 ± 15	450 ± 60	52 ± 20	471 ± 70	44 ± 8	420 ± 45	6 ± 2	88 ± 15	1	39 ± 8	0	0	0	0	0
10^{-3}	170 ± 30	12 ± 6	165 ± 30	8 ± 4	158 ± 25	3	33 ± 8	0	7 ± 1	0	5 ± 3	0	0	0	0	0
10^{-4}	9 ± 2	2	32 ± 7	2	1	0	3	0	1	0	0	0	0	0	0	0

[a] R, reference strain *H. pylori* ATCC 43629; C, clinical strain *H. pylori* 13 A. Data represent the results determined with suspensions after storage at 4°C in GESA transport medium over time. The number of CFU per plate are mean values of the results of three experiments performed in duplicate ± standard deviations (SD).

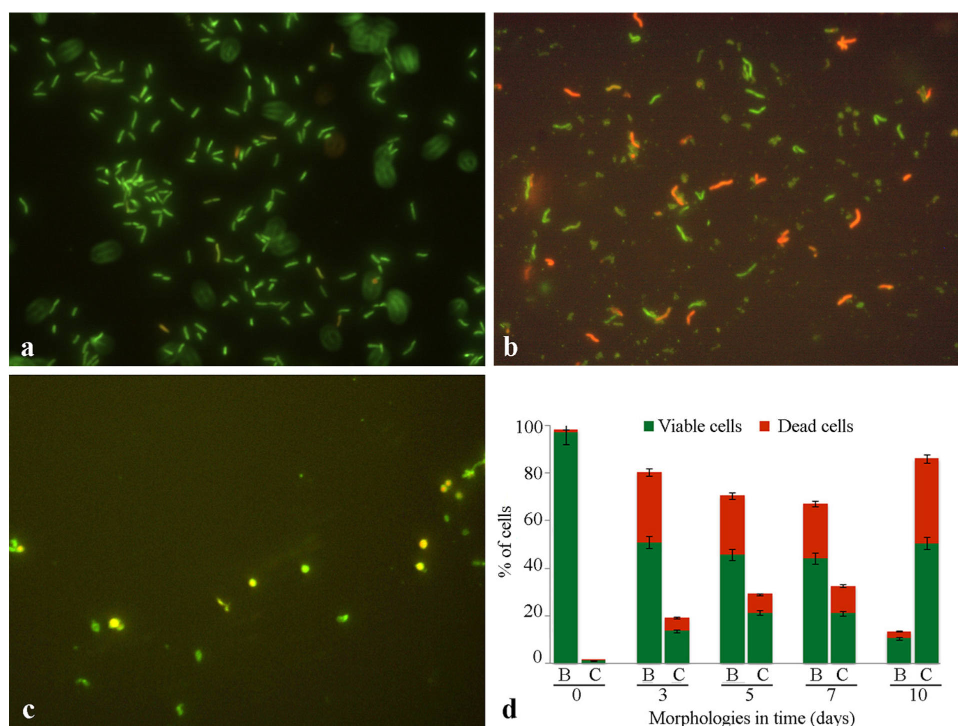

FIG 1 Morphology and viability of *Helicobacter pylori* stored in GESA transport medium over time. (a to c) Representative images of *H. pylori* ATCC 43629 after 1 (a), 7 (b), and 10 (c) days of inoculum Live/Dead staining. Magnification, ×1,000. (d) Percentages of spiral/bacillary (B) and coccoid (C) *H. pylori* cells and their viability, detected over time (in days). The values are the means ± standard deviations (SD) of the results obtained with both the reference and the clinical *H. pylori* strains.

port medium, over time. When 10 μl of the first microbial dilution was inoculated into GESA transport medium at 4°C, it was possible to recover *H. pylori* colonies at up to 10 days of storage. At this inoculum concentration and after 7 days, means of 150 ± 25 *H. pylori* ATCC 43629 colonies and 40 ± 7 *H. pylori* 13 A colonies were counted on AC medium, respectively. However, a significant reduction in the bacterial recovery rate was observed in the reference strain after 4 days of incubation (from 490 ± 70 to 88 ± 15, $P < 0.05$) and already after 1 day in the clinical isolate (from 106 ± 15 to 52 ± 20, $P < 0.05$) with a dilution of 10^{-2}.

In all cases, efficacy of recovery of microorganisms was obtained even at a very low inoculum concentration; with a dilution of 10^{-4} of the starting bacterial suspension, *H. pylori* ATCC 43629 was detected until the 4-day time point.

No significant differences in terms of colonies recovered were detected when GESA transport medium was stored at 4°C for 12 months before use.

The morphology and viability of *H. pylori* suspensions inoculated in GESA transport medium detected over time are shown in Fig. 1. The typical green spiral morphology was prevalent until the 7-day time point (Fig. 1a and b). Green viable cells were detectable after 10 days of storage also but were in their unculturable coccoid shape (Fig. 1c). Histograms in Fig. 1d express the percentages of viable and dead cells in both spiral/bacillary (B) and coccoid (C) morphologies. The recorded morphologies were similar for the reference and the clinical *H. pylori* strains.

Recovery of *H. pylori* from biopsy specimens. Biopsy specimens that were UBT positive were included in the recovery assay. *H. pylori* was recorded from both the antral and fundus specimens from GESA transport medium with a recovery rate of 90.66% in 68 of the 75 patients examined. Bacterial colonies were isolated after 3 to 4 days of biopsy culture, and the absence of contaminant colonies facilitated the subsequent antimicrobial testing. Among the 7 patients in whom *H. pylori* was undetected both in antrum and fundus, the bacterial recovery was compromised because of bacterial overgrowth due to forceps contamination in 3 cases, whereas no bacterial growth was detected in plated media in the remaining 4 cases.

Therefore, GESA is a standardized transport medium that ensures greater stability over time, preserving its features after 1 year of storage at 4°C.

Table 3 shows data from the positive *H. pylori* samples from multiple biopsy specimens taken from gastric antrum and fundus of 13 patients and stored until the 10-day time point and randomly processed. In all detected biopsy specimens and at each time point of recovery, cultivable *H. pylori* cells were detected and isolated. In each analyzed time of recovery, *H. pylori* colonies were suitable for subsequent antimicrobial testing or collecting for long-term storage.

DISCUSSION

Detection of *H. pylori* through cultural isolation represents a procedure that requires microbiological expertise, and it is significantly influenced by the conditions of transport of the biopsy specimen from the endoscopy unit to the laboratory. Moreover, bacterial isolation is expensive and requires invasive techniques with great variability in susceptibility and time of recovery of the bacteria (22).

TABLE 3 *Helicobacter pylori* isolation from biopsy samples stored at 4°C for up to 10 days in GESA transport medium and randomly processed

	Presence of *H. pylori* in sample from day:									
Biopsy sample	1	2	3	4	5	6	7	8	9	10
Antrum										
18 A 2011	+	NT[a]	+	NT	+	NT	NT	NT	+	NT
19 A 2011	+	+	NT	+	NT	NT	NT	NT	NT	+
20 A 2011	+	+	NT	NT	NT	NT	NT	+	NT	+
21 A 2011	+	NT	+	NT	NT	NT	NT	+	NT	+
22 A 2011	+	NT	NT	+	NT	+	NT	NT	NT	+
23 A 2011	+	NT	NT	NT	+	NT	+	+	NT	NT
24 A 2011	+	+	NT	NT	NT	+	NT	NT	NT	+
25 A 2011	+	NT	+	NT	NT	NT	NT	+	NT	+
1 A 2012	+	+	NT	NT	NT	+	NT	NT	NT	+
2 A 2012	+	+	NT	NT	NT	NT	+	NT	NT	+
3 A 2012	+	NT	NT	+	NT	+	NT	NT	NT	+
4 A 2012	+	NT	NT	+	NT	NT	+	NT	+	NT
5 A 2012	+	NT	+	NT	+	NT	NT	+	NT	NT
Fundus										
18 F 2011	+	NT	+	NT	NT	NT	+	NT	+	NT
19 F 2011	+	+	NT	NT	NT	+	NT	NT	NT	+
20 F 2011	+	NT	NT	+	NT	NT	NT	+	NT	+
21 F 2011	+	NT	+	NT	+	NT	NT	+	NT	NT
22 F 2011	+	+	NT	+	NT	NT	NT	NT	NT	+
23 F 2011	+	NT	NT	NT	NT	NT	+	+	NT	+
24 F 2011	+	NT	NT	NT	+	+	NT	NT	NT	+
25 F 2011	+	NT	+	NT	NT	NT	NT	+	NT	+
1 F 2012	+	+	NT	NT	+	NT	NT	NT	NT	+
2 F 2012	+	+	NT	NT	NT	NT	+	NT	NT	+
3 F 2012	+	NT	+	+	NT	NT	NT	NT	NT	+
4 F 2012	+	NT	NT	+	NT	+	+	NT	NT	NT
5 F 2012	+	NT	+	NT	+	NT	NT	+	NT	NT

[a] NT, not tested.

Despite these disadvantages, detection of the colonies represents the only methodology that allows testing for antimicrobial susceptibility and molecular analysis (22). In the last 2 decades, increases in drug resistance, particularly in some areas of the world (7, 23), strongly suggest the use of methods that incorporate antibiotic testing before therapy for effective management of *H. pylori* infections.

In our Italian area, the increase in levels of resistance, in particular to clarithromycin and fluoroquinolones (24, 25) and especially in cases of relapse, needs great attention; culture and, consequently, susceptibility testing represent, undoubtedly, the most correct way to administer a successful therapy for eradication, preventing emergence of multiresistant *H. pylori* strains.

GESA transport medium enables transportation of the gastric biopsy specimen from endoscopy to the microbiology laboratory, guaranteeing the long-term viability and cultivability of the microorganism.

This report highlights the benefits of this new patented medium for high-performance recovery of *H. pylori*.

Several other transport media have been described, such as Stuart's media (18, 26–28), serum-free transport medium with cyanobacterial extract (MH-CE) (20), brain heart infusion broth plus vancomycin, amphotericin B, and nalidixic acid (BHI-VAN) (29), and a commercial medium, Portagerm *pylori* (18, 26). The composition of each of experimental medium was not critical for the recovery of cultivable *H. pylori* strains within 1 day of storage, with good performance in bacterial isolation seen for each of them. When immediate culture was not feasible, few media were useful in prolonging survival of culturable *H. pylori* from cell suspensions and biopsy specimens (18, 29) with suitable yields of *H. pylori* recovery over time.

The composition of the proposed new semisolid medium allows long-term *H. pylori* recovery from both suspensions and gastric biopsy specimens; with respect to other proposed transport media (5, 17, 30), GESA shows a quantifiable bacterial recovery rate until the 10-day time point.

By the use of *H. pylori* suspensions in experiments, the microscopy fluorescence observations underline the preservation, over time, of a consistent part of the bacterial population with the spiral/bacillary viable *H. pylori* morphology useful for bacterial growth on cultural media. Storage at 4°C in GESA transport medium does not modify the typical cell morphology until the 7-day time point, thus guaranteeing bacterial recovery and, consequently, suitability for drug susceptibility testing and biomolecular analysis of gene targets of virulence factors. The loss in bacterial recovery after 10 days, with detection of few *H. pylori* CFU, was confirmed by the fact that the cells, which showed a marked morphological change from spiral to coccoid, despite the prevalent viability, were unculturable. These results underline the good performances of GESA medium. In a previous study, Vega et al. (20) reported recovery of bacterial cells in 5 of 7 *H. pylori* strains in MH-CE for up to 5 days of storage at 4°C, whereas other authors (28) reported *H. pylori* survival and cultivability for up to 3 days when inocula were stored in Stuart medium.

Regarding *H. pylori* recovery from biopsy specimens, we collected *H. pylori* colonies until 10 days of storage at 4°C, and, interestingly, saw no differences in the levels of colony recovery over time, thus supporting the hypothesis of the high performance of GESA transport medium in maintaining storage of viable and cultivable cells from gastric specimens. This is of particular interest when it is necessary to prolong the biopsy specimen storage with a guarantee of satisfactory bacterial isolation. Other selective transport media showed lower rates of recovery of *H. pylori*, with values of 76% for 5 days in BHI-VAN (29), 77% for 3 days in Portagerm *pylori* (18), and 61% for 4 days in MH-CE (20).

The better GESA performance in the rate of recovery in bacterial colonies from biopsy specimens than in bacterial colonies from suspensions was also noticed for other transport media (18, 19). In particular, Heep et al. (18) suggested that gastric tissue exerts a protective effect during cold storage. We suppose that the protective effect deriving from the adhesion of the microorganism to the gastric epithelial tissue can help the *H. pylori* to avoid responding to the stress condition by entering the viable but nonculturable (VBNC) state and becoming coccoid (11–14). Moreover, the formulation of GESA transport medium and the absence of antibiotics could favor the fast recovery of *H. pylori* colonies. In fact, we obtained bacterial isolation in a time period never exceeding 4 days in the absence of contaminant, allowing rapid bacterial susceptibility testing and a fast response for patients. These results could be attributed both to the formulation of the medium and to the absence of antibiotics that, even among those ineffective against *H. pylori*, could be capable of stressing the microorganism and inducing, in a part of bacterial population, a loss of cultivability. The GESA formulation could also avoid bacterial overgrowth, providing easy recovery of *H. pylori* colonies from biopsy specimens with very low rates of contamination until the 10-day

time point. Obviously, good practices with respect to biopsy specimen collection and the sterility of forceps are important factors for successful isolation.

GESA can be considered a standardized transport medium with high performance that optimizes the rate of recovery of *H. pylori* by culture.

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