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## Selective Antibody Response to *Streptococcus gallolyticus* Pilus Proteins in Colorectal Cancer Patients

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### Abstract

*Streptococcus gallolyticus* subsp. *gallolyticus* (previously called *Streptococcus bovis* biotype I) infections have long been associated with colorectal cancer (CRC). This work aimed to investigate the CRC-associated humoral immune response to four pilus proteins of this bacterium by newly developed ELISAs. Pilus proteins are interesting diagnostic targets as they are the building blocks of pilin-like structures that mediate bacterial virulence and are readily exposed to the host immune system upon infection. The presence of serum antibodies against these pilus proteins was evaluated in Dutch and American populations. These analyses showed that an immune response to these antigens was specific for clinical *S. gallolyticus* subsp. *gallolyticus* infections, but that increased serum antibody titers to multiple pilus proteins in single individuals were rarely observed. However, a multiplex approach based on antibody titers against any of these four antigens resulted in assay sensitivities between 16% and 43% for the detection of early-stage CRC. Together these findings underscore the potential of a multi-antigen approach to complement diagnosis of *S. gallolyticus* subsp. *gallolyticus*-associated CRC.

### Introduction

The human gastrointestinal tract is the habitat for a large and dynamic bacterial community, which is essential for digestion of food and the control of intestinal epithelial homeostasis (1, 2). Remarkably, although hundreds of bacterial species reside in the human intestinal tract, only the opportunistic pathogen *Streptococcus gallolyticus* subsp. *gallolyticus* (*S. bovis* biotype I), seems to benefit from the presence of premalignant colonic lesions to invade the human body (2–7). In this respect, *S. gallolyticus* subsp. *gallolyticus* can be regarded as a whistle-blower for colorectal cancer (CRC) as multiple studies showed that a (precursor of) CRC is detected in 33% to 100% of the individuals that undergo full bowel examination following diagnosis of this infection. Notably, our recent meta-analysis showed

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#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

that this percentage is far above the prevalence of this disease in the asymptomatic age-matched population (8). *S. gallolyticus* subsp. *gallolyticus* is a known causative agent for infective endocarditis (IE), however, due to its mild virulence characteristics this bacterium can only establish a clinical infection in patients with preexisting heart valve abnormalities. Molecular studies suggested that *S. gallolyticus* subsp. *gallolyticus* is relatively invisible for the innate immune system due to its inert surface structure (9, 10; our unpublished observations), which implies that *S. gallolyticus* subsp. *gallolyticus* may cause subclinical infections in a substantial part of CRC patients (11). The latter idea was supported by our previous finding that the humoral immune response against the ribosomal protein L7/L12 from *S. gallolyticus* subsp. *gallolyticus* was significantly increased in early-stage CRC patients (12–14), which is indicative for an increased exposure to this bacterium. However, a drawback of this approach was the fact that the high conservation of this antigen in the bacterial kingdom was associated with a considerable amount of cross-reactivity in the immunoassay (14).

This study aimed at the development of new ELISAs exploiting antigens that are specific for *S. gallolyticus* subsp. *gallolyticus* strains. These candidate antigens concerned 4 cell wall peptidoglycan-anchored proteins that form pilin-like structures on the *S. gallolyticus* subsp. *gallolyticus* cell surface (15). Two of these proteins, annotated Gallo2178 (major pilin) and Gallo2179 (collagen-binding adhesin) are encoded by the *pil1* locus that also encodes a sortase (Gallo2177) which is specifically responsible for the polymerization of these 2 LP×TG into a pilus structure. The *pil1* locus is present in the majority of clinical *S. gallolyticus* subsp. *gallolyticus* IE isolates and involved in binding to collagen type I, biofilm formation, and virulence in a rat model of experimental endocarditis (to be published elsewhere). Interestingly, collagen I-binding capacity has also been proposed as a distinguished virulence feature of *S. gallolyticus* subsp. *gallolyticus* strains to facilitate its adherence to premalignant colonic sites (9). Collagen binding is likely to be mediated by Gallo2179, which contains a collagen-binding domain. The other 2 candidate antigens, Gallo1569 and Gallo2039, are major pilins related to Gallo2178, but encoded by the *pil2* and *pil3* operons, respectively (16). The *pil2* operon has a low conservation among *S. gallolyticus* subsp. *gallolyticus* strains, whereas homologous *pil3* operons can also be found in *Streptococcus infantarius* subsp. *infantarius* strains.

Our current data showed that ELISAs with these 4 antigens were indeed specific for *S. gallolyticus* subsp. *gallolyticus* infections. Furthermore, our data showed a highly selective humoral immune response to these antigens in CRC patients. However, a multimarker approach could identify a substantial number of these patients. This finding argues in favor of developing extended multiplex assays based on specific antigens from CRC-associated bacteria as screening tool for CRC.

## Materials and Methods

### Patient material

Blood samples were derived from the same collections as used before in our studies (14). However, here, we primarily focused on the early stages of CRC (i.e., colorectal adenomas and local stage of colorectal cancer). Serum samples from 37 CRCs, 12 polyp patients (6 adenomas, 2 villous adenomas, and 4 undefined polyps), and 15 patients with a clinical bacterial infection [*Escherichia coli* (3), *Klebsiella pneumoniae* (3), *Salmonella typhimurium* (3), *Streptococcus pneumoniae* (3), or *S. gallolyticus* subsp. *gallolyticus* (3; CRC diagnosed in 1 patient) who had been admitted to the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands) were used. Patients suffering from bacterial infections were recognized as such by a positive blood culture and routine microbial typing. As control, serum samples from 27 healthy blood donors (>50 years), who did not undergo

colonic evaluation, were used. In addition, plasma samples from 33 CRC, 11 polyp patients, and 47 healthy controls who participated in a population-based case-control study in Metropolitan Detroit, were included as a second independent study population. CRC samples concerned localized disease (stage I or II), with the exception of 7 Detroit cases with unknown stage. All cases in both the Nijmegen and Detroit population underwent colonic evaluation. The use of the samples was approved by the Medical Ethical Committee of Nijmegen/Arnhem (#2006/078) and Wayne State University Human Investigation Committee (#0409000504); informed consent was obtained when required. Serum and plasma samples were stored at  $-80^{\circ}\text{C}$  until use.

### Antigen overproduction and purification

DNA fragments internal to *gallo2179*, *gallo2178*, *gallo2039* and *gallo1569* were produced by PCR using genomic DNA of UCN34 strain as template and the primers described in Supplementary Table S1. These DNA fragments were digested with the appropriate enzymes (*NheI/NdeI* and *BamHI*) and cloned into pET28-a(+) (Novagen). The resulting plasmids were introduced into *E. coli* strain DH5 $\alpha$  for sequence analysis or BL21(IDE3) for protein expression. For overproduction, cells were grown aerobically at  $37^{\circ}\text{C}$  until  $\text{OD}_{600\text{nm}} = 0.4$  after which recombinant gene expression was induced by 1 mmol/L isopropyl  $\beta$ -D-1-thiogalactopyranoside. Recombinant His-tagged antigens were purified (>95%) under native conditions by affinity chromatography using the Nickel-NTA columns according to the manufacturers' recommendations (Novagen). Cell lysates and isolated protein fractions were analyzed by SDS-PAGE, and accurate protein concentrations were determined with the BCA system (Pierce) prior to use in ELISAs.

### ELISA measurements

Antigens ELISAs were built and conducted as described before (14). A few improvements were made as we observed that background levels due to nonspecific binding of serum immunoglobulins to the ELISA plate could vary significantly between samples. First, after antigen (AG) coating for at least 18 hours at  $4^{\circ}\text{C}$ , the well was extensively blocked by 1% bovine serum albumin (BSA) in PBS-Tween20 (0.1%) for 2 hours at  $37^{\circ}\text{C}$ . In addition, for each antigen-coated well, a duplicate well on the same plate was incubated in coating buffer without antigens and subsequently blocked with 1% BSA (blank). Finally, 1% BSA was added to all incubation buffers that were used during the ELISA measurements. The optical density of horseradish peroxidase-converted 3,3',5,5'-tetramethylbenzidine substrate was quantified at a wavelength of 450 nm in a spectrophotometer. Samples were measured in duplicate and titers of a specific sample were calculated as the mean  $\text{OD}_{450\text{AG}} - \text{OD}_{450\text{blank}}$  and expressed as arbitrary *S. gallolyticus* units (SGU) based on a reference sample from a *S. gallolyticus*-infected patient that was measured in every plate. Titers were set to zero in case of negative values.

## Results

### Specific humoral immune response to *S. gallolyticus* pilus proteins

The current study aimed at the development of new ELISAs exploiting antigens that are specific for *S. gallolyticus* subsp. *gallolyticus* strains (further designated as *S. gallolyticus*; ref. 8). To this purpose, purified pilus proteins Gallo1569, -2039, -2178, and -2179 from *S. gallolyticus* were used as immunoglobulin G (IgG)-capturing antigens in an ELISA setup. To evaluate whether an antibody response to these antigens was specific for *S. gallolyticus* infections, the corresponding serum IgG levels were determined in serum from patients exhibiting various clinical bacterial infections. As shown in Fig. 1, the anti-Gallo2178 and -2179 IgG levels in serum from 2 out of 3 *S. gallolyticus*-infected patients were several orders of magnitude higher than those in control patients diagnosed with *E. coli*, *K.*

*pneumoniae*, *S. typhimurium* or *S. pneumonia* infections. For the Gallo2039 antigen, this was only observed in 1 patient. The anti-Gallo1569 IgG levels were mildly increased in serum from all 3 *S. gallolyticus*-infected individuals, but they displayed superior discrimination between *S. gallolyticus* and the other clinical bacterial infections. These data clearly illustrate that an antibody response to these antigens could be a useful clinical marker of *S. gallolyticus* infection. However, it also points out that this humoral immune response has a high interindividual variation.

### Selective humoral immune response to *S. gallolyticus* pilus proteins in CRC patients

To investigate whether subclinical infections with *S. gallolyticus* can be monitored in CRC patients by the developed pilus ELISAs, serum/plasma from polyp, early-stage CRC patients, and asymptomatic age-matched individuals from the Nijmegen (serum) and Detroit (plasma) populations (14) were measured. As shown in Fig. 2, the IgG titers in serum/plasma of CRC patients never reached those observed in patients with a clinical *S. gallolyticus* infection. In addition, no significant differences were observed between the polyp, CRC, and control groups. However, with a few exceptions, the highest IgG titers were observed in serum/plasma from CRC patients.

### The potential of *S. gallolyticus* pilus antigens for CRC diagnostics

To assess to which extent an antibody response to *S. gallolyticus* pilus proteins can be instrumental in the diagnosis of CRC, a simple strategy was employed in which the highest IgG level in the control group was used as cutoff value for the diseased groups. This implicates that the assay has a 100% specificity as all controls are classified as not diseased. Table 1A shows that no single antigen has clear superior diagnostic value over another, and the sensitivity for polyps or CRC is only marginal when assessed for single pilin antigens. The highest sensitivity for CRC (4 positive identifications; 9%) was obtained for IgG titers against Gallo2178 in the Detroit population. When a combined approach was used in which antibody titers against any one of the 4 pilus antigens was considered to be diagnostic for polyps or tumors, the sensitivity for both the Nijmegen and Detroit populations was increased to 16%. As about 22% of the asymptomatic elderly population carries polyps (17), which may form a portal of entry for *S. gallolyticus* the sensitivity of the ELISAs for CRC was calculated using lower cutoff levels (i.e., 95 percentile in each control group), that correspond to an overall specificity of about 78%. As shown in Table 1B, this yielded an increased sensitivity of 20% in the Nijmegen population and 43% in the Detroit population. Figure 3 shows that most of these positive cases were diagnosed by increased IgG titers against only one of the four antigens. An immune response to 2, 3, or 4 antigens was only detected in 15%, 4%, and 2% of the cases, respectively. These data exemplify the added value of a multimarker approach for the detection of CRC based on bacterial antigens.

## Discussion

Inspired by the results of our previous studies that were indicative of an increased exposure to *S. gallolyticus* antigens in early-stage CRC patients (14), we here aimed at the development of ELISAs for the specific detection of human infections with this bacterium and the assessment of their utility for CRC diagnosis.

Four pilus proteins from *S. gallolyticus* were purified and used to develop ELISAs to measure the corresponding serum IgG levels. Pilus proteins are surface exposed and carry out key functions during infection and are quite specific for *S. gallolyticus*. This is for instance reflected by the unique collagen-binding capacity of this bacterium mediated by Gallo2179, which is absent in the closely related bacteria *S. infantarius* subsp. *infantarius* and *S. gallolyticus* subsp. *macedonicus* (9). Notably, homologues of Gallo2039 are also

found in related species, which implies that an anti-body response against this antigen could be associated with a decreased specificity for *S. gallolyticus* subsp. *gallolyticus* infections. Interestingly, the ELISA based on Gallo2039 did indeed yield the highest average IgG levels, which was most prominently observed in the Detroit population (Fig. 2). For the other 3 antigens, the corresponding IgG levels were in most individuals not significantly increased compared to the background (blank) level, which underscores the specificity of the strong response that was observed in a minority of CRC cases.

The responses in patients with known clinical *S. gallolyticus* infections show a variable IgG response (Fig. 1), which could be due to the individual status of the immune system and/or strain-specific differences in pilus expression (to be published elsewhere). It should be realized that bacterial pilin expression and the corresponding immune response are likely to be different during local infiltration of the bowel wall (subclinical infection) in CRC patients. However, on the basis of 3 *S. gallolyticus*-infected patients, we may estimate that no more than 66% of infected patients can be distinguished as such based on increased IgG levels to any of the 4 pilus antigens tested. The maximum sensitivity for the diagnosis of CRC is further decreased by the fact that the presence of *S. gallolyticus* in the large bowel of CRC patients may not exceed 50% (18), while the presence of genomic DNA of this bacterium in tumor tissue samples was reported to be around 35% (19). On the basis of these findings, the estimated limit of the current 4 *S. gallolyticus* pilus antigen-based approach for CRC detection would lay between 35% and 50%. Promisingly, our current data are indicative of an assay sensitivity between 20% to 43%, assuming that it is also diagnostic for adenomatous polyps with a prevalence of about 22% in the general elderly population (see Table 1).

Evidently, the above assumptions need to be validated with a properly designed study using serum samples from colonoscopy-controlled samples from healthy individuals, CRC patients as well as from patients with inflammatory bowel disease (IBD), who have reported bowel colonization rates in between healthy controls and CRC patients (4). Furthermore, the reassessment of bowel colonization of *S. gallolyticus* and its association with IBD, CRC, and adenoma tissues are both subjects of our ongoing investigations. To further increase the detection of CRC with this antigen approach, additional *S. gallolyticus*-specific antigens should be added to the here presented panel of 4 antigens, together with antigens from other CRC-associated bacteria and/or CRC tumor antigens (20, 21). Finally, the specificity of this multi-antigen approach should be validated in serum from patients having clinical infections with related Gram-positive pathogens (e.g., enterococcal infections), which is also among the subjects of our ongoing investigations. Nevertheless, our current proof of concept study clearly shows that *S. gallolyticus* pilus proteins are promising antigens to aid in the immunodiagnosis of *S. gallolyticus*-associated CRC.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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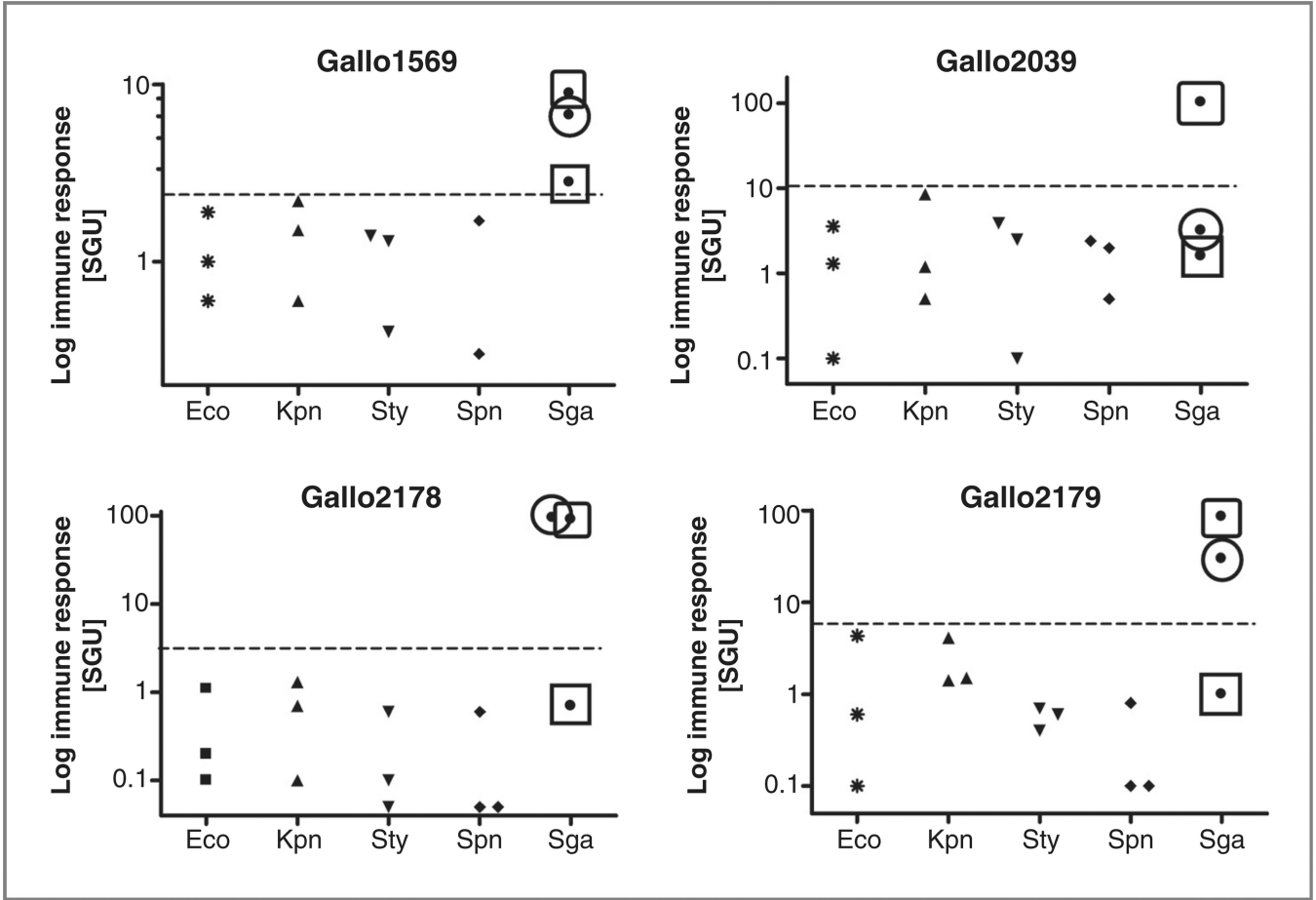
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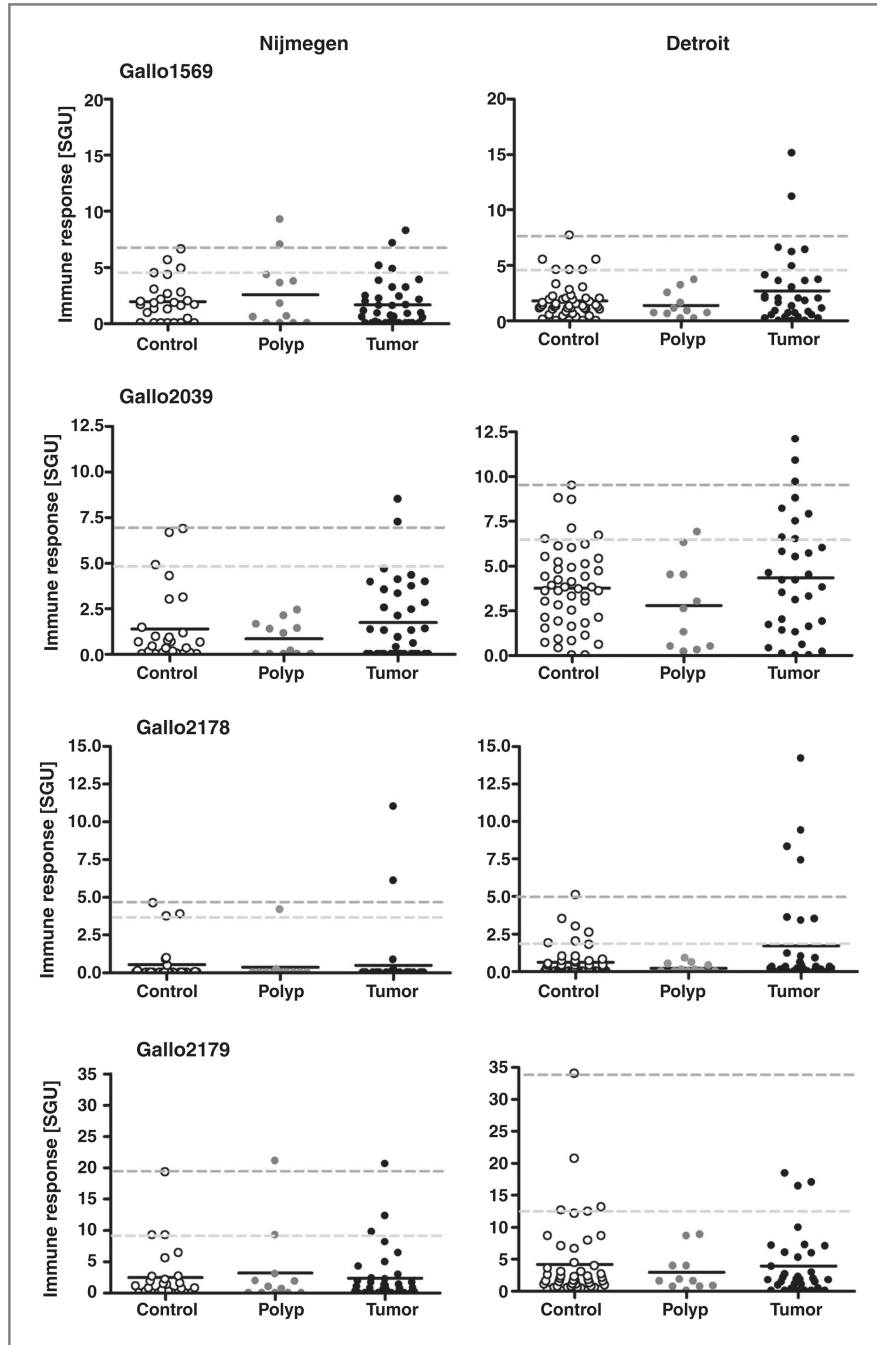
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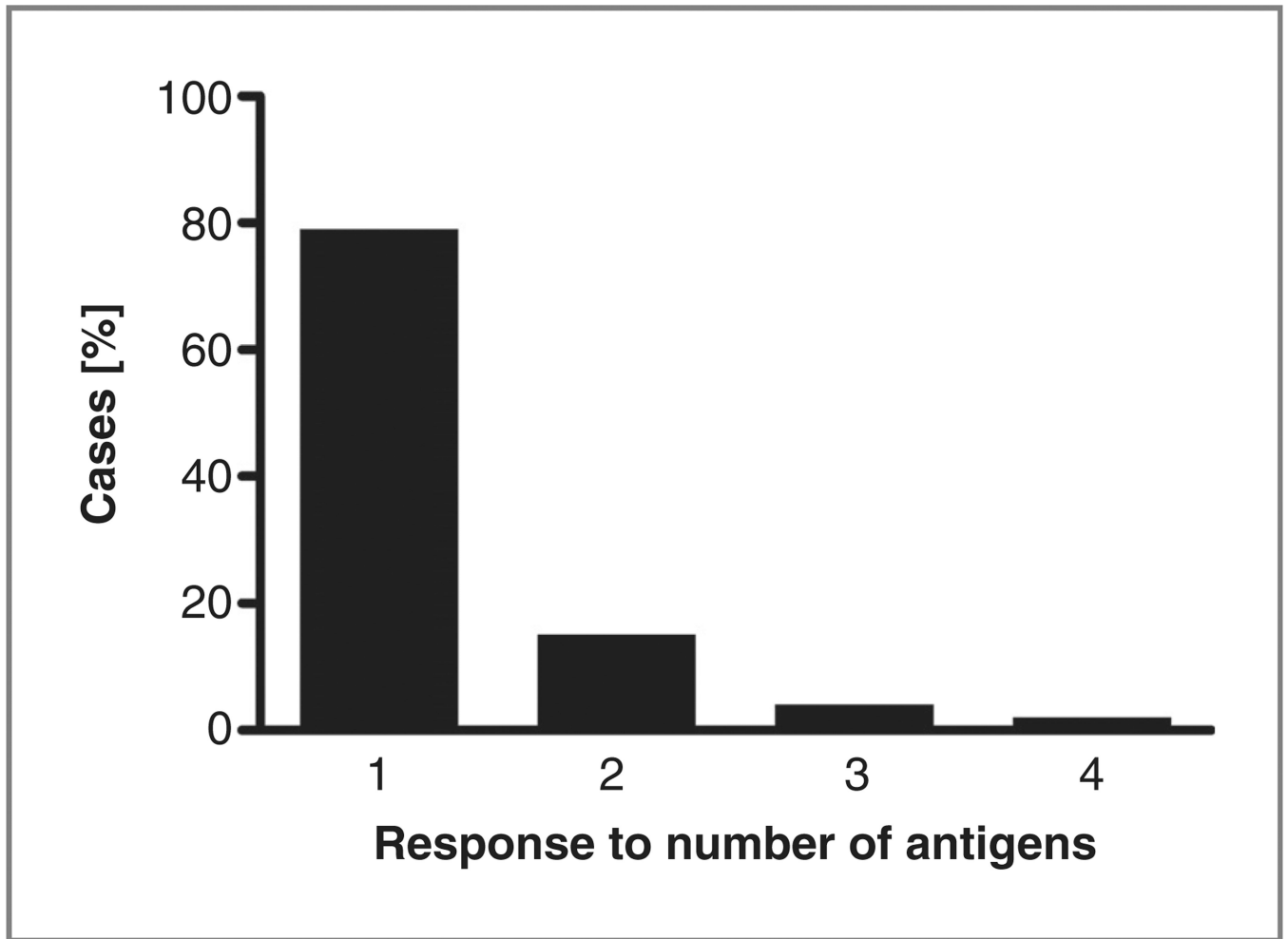
**Figure 1.** Pilin antibody levels in patients with systemic bacterial infections. Anti-Gallo1569, -Gallo2039, -Gallo2178, and -Gallo2179 IgG levels were measured in serum samples from patients with clinical infections with *E. coli* (Eco;  $n = 3$ ), *K. pneumoniae* (Kpn;  $n = 3$ ), *S. typhimurium* (Sty;  $n = 3$ ), *S. pneumoniae* (Spn;  $n = 3$ ), or *S. gallolyticus* (Sga;  $n = 3$ ). Anti-pilin IgG levels were expressed as arbitrary SGU. The data from *S. gallolyticus*-infected patients are differentially marked to allow assessment of the intraindividual differences in the responses to these 4 antigens.





**Figure 2.** Pilin antibody levels in patients with CRC and asymptomatic controls. Anti-Gallo1569, -Gallo2039, -Gallo2178, and -Gallo2179 IgG levels were measured in serum (Nijmegen population) and plasma samples (Detroit population) from polyp and CRC patients and in samples from asymptomatic age-matched controls. Anti-pilin IgG levels were expressed as arbitrary SGU; median levels are indicated. Nijmegen population: controls ( $n = 27$ ), polyp patients ( $n = 12$ ), and CRC patients ( $n = 37$ ). Detroit population: controls ( $n = 47$ ), polyp patients ( $n = 11$ ), and CRC patients ( $n = 33$ ). Cutoff values used to calculate assay specificity as indicated in Table 1 are indicated as dashed lines; upper line reflects 100% specificity (no positive controls) and lower line reflects 78% specificity (i.e., the 95

percentile in each control group) which adjusts for a 22% incidence of polyps in the control group.



**Figure 3.** Distribution of anti-pilin IgG levels. From the cases that were positively diagnosed at a specificity level of approximately 78% (Table 1B), the number of antigens to which IgGs were detected was plotted against percentage of cases.

**Table 1**

Sensitivity and specificity of *S. gallolyticus* pilin-based ELISAs for the detection of CRC<sup>a</sup>

Antigen	Nijmegen (49 cases and 27 noncases)				Detroit (44 cases and 47 noncases)			
	Cutoffs	Positive cases/ noncases <sup>b</sup>	Sensitivity (%)	Specificity (%)	Cutoffs	Positive cases/ noncases <sup>b</sup>	Sensitivity (%)	Specificity (%)
<b>A</b>								
Gallo1569	6.6	3/0	6	100	7.7	2/0	5	100
Gallo2039	6.9	2/0	4	100	9.5	3/0	7	100
Gallo2178	4.6	2/0	4	100	5.1	4/0	9	100
Gallo2179	19.2	2/0	4	100	34.0	0/0	0	100
Any of the above		8/0	<b>16</b>	<b>100</b>		7/0	<b>16</b>	<b>100</b>
<b>B</b>								
Gallo1569	4.9	5/2	10	93	4.6	6/4	14	92
Gallo2039	4.9	2/2	4	93	6.7	8/4	18	92
Gallo2178	3.7	3/2	6	93	2.0	7/4	16	92
Gallo2179	9.2	5/2	10	93	12.4	3/4	7	92
Any of the above		10/6	<b>20</b>	<b>78</b>		19/10	<b>43</b>	<b>78</b>

<sup>a</sup> CRC includes both polyps and tumor cases; the lower cutoff value in B represents the 95 percentile in each control group.

<sup>b</sup> Number of cases and noncases above the specified ELISA cutoff.