

Adherence of Group B Streptococci to Human Rectal and Vaginal Epithelial Cell Lines in relation to Capsular Polysaccharides as well as Alpha-like Protein Genes – Pilot Study

MALGORZATA BODASZEWSKA-LUBAS^{1*}, MONIKA BRZYCHCZY-WLOCH¹, PAWEŁ ADAMSKI¹,
TOMASZ GOSIEWSKI¹, MAGDALENA STRUS¹ and PIOTR B. HECZKO¹

¹Department of Bacteriology, Microbial Ecology and Parasitology, Chair of Microbiology
Jagiellonian University Medical College, Cracow, Poland

²Institute of Nature Conservation, Polish Academy of Sciences, Cracow, Poland

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Abstract

Streptococcus agalactiae (Group B Streptococci, GBS) constitutes a risk factor for infections of the newborns born by colonized mothers. The adherence of GBS to epithelial cells has been proved to be an important factor in the colonization of mucus membranes of both human rectum and vagina. The objective of the study was to assess the adhesion of the selected GBS strains to the human colon adenocarcinoma cell line (HT-29) and human epidermoid vulvo-vaginal cells (A-431) in relation to the capsular polysaccharides and alpha-like protein genes. GBS strains from the human sources belonging to Ia, Ib, II, III and V serotypes possessing different surface alpha-like protein genes such as the *alp 2*, *alp 3*, *bca*, *epsilon* and *rib* in the conventional adherence assay were examined. The adherence of GBS strains to the HT-29 cell line was considerably higher than to the A-431 cell line. For GBS serotype Ia and III, a significant difference between the adhesion to the HT-29 and A-431 cell lines was presented. The adhesion of GBS strains to the HT-29 cell line depended on alpha-like protein genes. The most adhesive ones were the GBS strains containing the *rib* and *alp 2* genes. The adherence of GBS strains to the A-431 cell line depended on both their serotype and alpha-like protein genes. Serotype III adhered to the A-431 cells most tightly, particularly the strains containing the *rib* and *alp 2* genes. GBS strains containing the *rib* gene adhered to the HT-29 and A-431 cell lines more firmly than GBS strains containing other alpha-like protein genes.

Key words: adherence, alpha-like protein genes, capsular polysaccharides (CPS), cell lines, group B Streptococci (GBS)

Streptococcus agalactiae (Group B Streptococci, GBS) is a bacterium colonizing mainly the epithelial cells of the lower gastrointestinal tract and the vagina. The epidemiological data confirms the relation between the rectal and vaginal colonization of *S. agalactiae* in pregnant women and infections of the infants triggered by this bacterium. During the delivery there is a danger of infant colonization with GBS coming from the mother's microflora of the vagina or cervical canal through vertical transmission. Group B streptococci colonizes the infant's oral cavity and afterwards the respiratory tract and gastrointestinal tract. The risk of GBS transmission equals 70%. *S. agalactiae* may trigger the infant's infections which can be divided, depending on time of occurrence after the delivery, into: early onset diseases (EOD) revealing mainly in the form of pneumonia and late onset diseases (LOD) most frequently manifesting in the form of meningitis and sepsis. (Schrag *et al.*, 2000; Zaleźnik *et al.*, 2000; Verani *et al.*, 2010).

Pathogenesis of the infections triggered by GBS is a process consisting of several phases. The first one is undoubtedly bacterial adherence to the cells of the host conditioning colonization and invasion of the bacteria and influencing the course of the infection (Sohn *et al.*, 2006). The adherence of GBS to epithelial cells of, among others: the vagina, rectum and lung progresses as a result of the interaction between the bacterial adhesins and extracellular matrix proteins (Kim *et al.*, 2010).

The alpha-like proteins (the Alp family) constitute the best known and described GBS surface proteins. In this class of proteins we can differentiate the following: the Alp 2, Alp 3, Alp 4, Alpha C, Epsilon and Rib which are encoded by allelic genes, respectively: *alp 2*, *alp 3*, *alp 4*, *bca*, *epsilon* and *rib* and possess the tandem repeated region in their structure reflected in the structure of a protein molecule as well as conservative N-terminus and C-terminus. (Creti *et al.*, 2004; Gherardi *et al.*, 2007; Persson *et al.*, 2008; Lachenauer *et al.*, 2000).

* Corresponding author: M. Bodaszewska-Lubas, Department of Bacteriology, Microbial Ecology and Parasitology, Chair of Microbiology, Jagiellonian University Medical College; 31-121 Cracow, Poland, 18 Czysa Street; phone: +48 12 633 60 33; fax: +48 12 423 39 24; e-mail: mbodaszewska@gmail.com

On the surface of GBS cells there are also other factors which do not function as adhesins but can influence the course of bacterial adherence to the epithelium. There exist contrary theories concerning the effect of capsular polysaccharides (CPS) on the GBS adherence to the host cells. There is a probability that CPS can insignificantly increase the bacterial adhesion capacity (Lione *et al.*, 2005). Presumably, this can be related to the spacious effect of CPS on the process of interaction between the adhesion and the receptor on the surface of epithelial cells or repulsion of negatively-charged molecules of the sialic acid from the surface of the epithelial cells. Capsular polysaccharides condition the affinity of GBS strains to specific serotypes on the basis of differences in their structure. We can differentiate ten serotypes of *S. agalactiae*: Ia, Ib, II, III, IV, V, VII, VIII and serotype IX described in the recent years (Harrison *et al.*, 1998; Hickman *et al.*, 1999; Slotved *et al.*, 2007).

The objective of this work was to evaluate and compare the adherence capacities of selected *S. agalactiae* strains to the human colon adenocarcinoma cell line (HT-29) and the human epidermoid vulvo-vaginal cells (A-431) as well as to prove the relation between the GBS adherence to the examined tissue lines and affiliation of the strains to serotypes (Ia, Ib, II, III, V) and the genes encoding the alpha-like proteins (*alp 2*, *alp 3*, *bca*, *epsilon*, *rib*).

S. agalactiae strains came from the own collection of the Department of Bacteriology, Microbial Ecology and Parasitology, Chair of Microbiology, Jagiellonian University, Medical College. They were collected within the framework of the projects financed by the Ministry of Science and Higher Education no. 3 PO5E 084 25 as well as statutory research no. K/ZDS/000648, which were conducted upon consent of the Bioethics Committee of the Jagiellonian University Medical College no. KBE/267/B/2002 and no. KBET/143/B/2007. The examined GBS strains (n = 26) were isolated both from unpregnant women and pregnant women as well as newborns. The reference strains such as: *S. agalactiae* DSMZ 2134 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ), *S. agalactiae* ATCC 12403 (American Type Culture Collection, ATCC) and *S. agalactiae* ATCC BAA-611 (ATCC) constituted the control group.

GBS strains represented the most common serotypes (Ia, Ib, II, III and V) marked with the use of a multiplex-PCR reaction in accordance with the procedure of Poyart *et al.* (2007). They also possessed genes of various surface proteins from the alpha-like family (*alp 2*, *alp 3*, *bca*, *epsilon*, *rib*) marked with the application of the multiplex-PCR method pursuant to the procedure described by Creti *et al.* (2004) and Gherardi *et al.* (2007). The diversity and number of alpha-like

protein genes among five serotypes GBS strains (no. 26) and GBS standard strains (no. 3) are shown in Table I.

The HT-29 cell culture was conducted in DMEM medium (Ludwik Hirszfild Institute of Immunology and Experimental Therapy, Polish Academy of Science, IITD PAN) whereas the A-431 cell culture was carried out in RPMI 1640 medium (IITD PAN) with the addition of 10% inactivated (30 minutes, 56°C) fetal bovine serum (SIGMA) as well as Penicillin-Streptomycin antibiotic mixture (SIGMA) at the temperature of 37°C in the atmosphere of increased humidity and 5% carbon dioxide concentration (HERACELL CO2 Incubator, Thermo Scientific). In order to conduct an experiment the HT-29 cell suspension of the density of 4×10^4 cell/ml and the A-431 cell suspension of the density of 5×10^5 cell/ml were cultured in 24-well plates (Techno Plastic Products AG) on the bottom of which

Table I
Diversity and number of alpha-like protein genes among five serotypes GBS strains (no. 26) and GBS standard strains (no. 3).

No.	GBS strains	Sero-type	Alpha-like protein gene	Investigated group
1	K-A5	Ib	<i>bca</i>	unpregnant women
2	K-A2	III	<i>rib</i>	
3	K-A6	Ia	<i>epsilon</i>	
4	K-A15	II	<i>bca</i>	
5	K-A18	Ia	<i>epsilon</i>	
6	K-A17	V	<i>alp2</i>	
7	K-A14	V	<i>alp3</i>	
8	K-B10	Ib	<i>epsilon</i>	
9	K-B18	Ia	<i>epsilon</i>	
10	K-B14	III	<i>rib</i>	
11	K-B15	Ia	<i>bca</i>	
12	K-B11	II	<i>rib</i>	
13	KC-A5	Ia	<i>epsilon</i>	pregnant women
14	KC-A11	III	<i>alp2</i>	
15	KC-A13	Ia	<i>epsilon</i>	
16	KC-B6	Ia	<i>epsilon</i>	
17	KC-B11	III	<i>rib</i>	
18	KC-B13	III	<i>alp2</i>	
19	N-A2	II	<i>rib</i>	newborns
20	N-A3	II	<i>rib</i>	
21	N-A9	III	<i>bca</i>	
22	N-A13	Ib	<i>bca</i>	
23	N-A10	III	<i>rib</i>	
24	N-B3	Ia	<i>epsilon</i>	
25	N-B4	III	<i>rib</i>	
26	N-B13	III	<i>alp2</i>	
27	DSMZ 2134	II	<i>rib</i>	standard GBS strains
28	ATCC 12403	III	<i>alp2</i>	
29	BAA-611	V	<i>rib</i>	

microscopic cover glasses of the diameter of 12 mm (GREENPOL) had been placed earlier. To obtain a monolayer culture of the HT-29 cell line it was carried out on the plates for the period of 20 days and the culture medium was changed every day. The A-431 cell culture on the plates was continued for the period of 48 hours (Strus *et al.*, 2001).

In order to assess vitality and density of the examined tissue lines, the cells were trypsinized with the use of 25% trypsin solution (SIGMA) and afterwards they were stained with trypan blue (SIGMA) and counted in Buerker chamber. The density of the A-431 cell line and the HT-29 cell line used for the experiment was 1×10^6 cell/ml. The vitality of both tissue lines was 80%.

GBS strains were cultured in TSB (Tryptic Soy Broth, DIFCO Laboratories) for the period of 18 hours under aerobic condition at the temperature of 37°C (Incubator CLN ECO, POL-ECO Aparatura). On completion of incubation 18-hour GBS cultures were centrifuged (10 minutes, 3000 rpm) (SIGMA), washed three times with PBS without Ca^{2+} and Mg^{2+} (IITD PAN) and subsequently suspended in the medium mixture in ratio 1:1 of TSB:DMEM or TSB:RPMI 1640 depending on the requirement for tissue culture which participated in the experiment. The density of the obtained bacterial suspension was assessed with the use of spectrophotometric measurement (Spectrophotometer UV/VIS V-550, Jasco) at wavelength of 600 nm within the range of 0.6–0.7 and it was 1×10^8 c.f.u./ml (colony forming units per one millilitre).

The experiment was made by connecting the bacterial culture of the density of 1×10^8 c.f.u./ml with A-431 cell culture and the HT-29 cell culture of the cell densities of 1×10^6 /ml in ratio 1:100. The strains of *S. agalactiae* DSMZ 2134, *S. agalactiae* ATCC 12403 and *S. agalactiae* ATCC BAA-611 constituted the positive control whereas the negative one was PBS without Ca^{2+} i Mg^{2+} . After 30 minutes of incubation at the temperature of 37°C in the atmosphere of increased humidity and 5% carbon dioxide concentration the culture with adherent GBS cells was washed three times with PBS, fixed in 3.7% formaldehyde (POCH) and stained with the use of the Gram method (ANALAB). The adherence was quantitatively determined by counting GBS cells in 20 randomly selected fields of vision. The mean (\bar{x}) for 20 fields constituted the final result (Strus *et al.*, 2001).

To analyse the results the following techniques in the inductive statistics were applied: analysis of variance (ANOVA) and a supplementary post hoc analysis with the Tukey-Kramer test (Sokal and Rolf, 1995; Ferguson and Takane, 2009). If the distribution of numeric variable differed significantly from Gaussian curve the non-parametric alternatives to the above-mentioned tests were applied: the Kruskal-Wallis test as an alternative to ANOVA and Steel test instead of the Tukey-Kramer

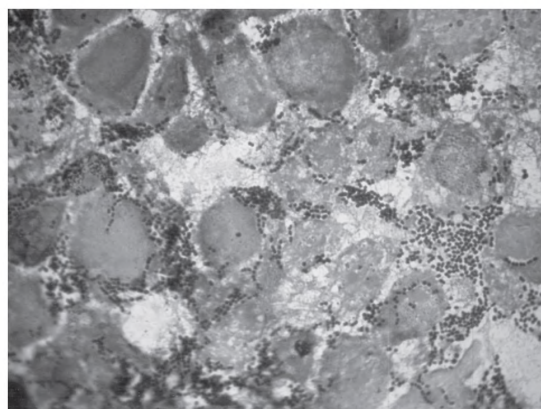


Fig. 1. Microscopic image of the A-431 cell line with adherent *S. agalactiae* ATCC BAA-611 strain (serotype V, *rib* gene).

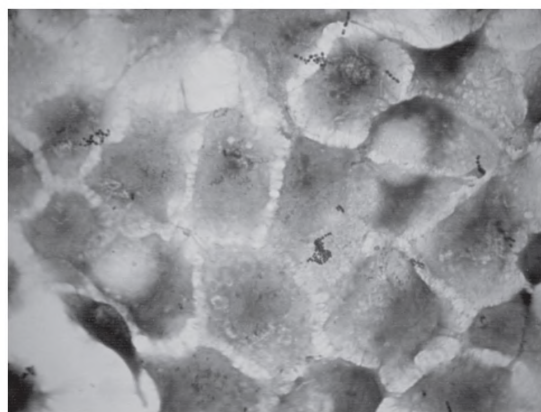


Fig. 2. Microscopic image of the A-431 cell line with adherent *S. agalactiae* ATCC BAA-611 strain (serotype V, *rib* gene).

test. The statistical procedures were conducted with JMP 7.01 (SAS Institute Inc.) (Stevens, 1989).

The adherence of selected 26 GBS strains and reference strains of *S. agalactiae* to the cell line of HT-29 human colon adenocarcinoma as well as the A-431 human epidermoid vulvo-vaginal cells was examined (Fig. 1, Fig. 2).

All the bacterial strains of *S. agalactiae* selected for the experiment adhered to both HT-29 and A-431 cell lines. There were statistically significant differences between *S. agalactiae* strains of the following serotypes: Ia, Ib, II, III and V ($P=0.0460$) as well as between GBS strains with specific alpha-like protein genes (*alp 2*, *alp 3*, *bca*, *epsilon*, *rib*) ($P=0.0038$) in adherence to both HT-29 and A-431 cell lines which would confirm the relation between the adherence of GBS and capsular polysaccharides (Fig. 3) as well as alpha-like protein genes (Fig. 4). Further analysis of the obtained results displayed statistically significant differences in the adherence to the HT-29 and A-431

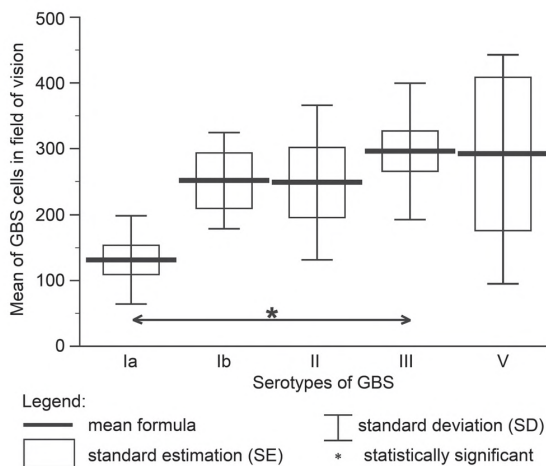


Fig. 3. The comparison of adherence of *S. agalactiae* strains belonging to the following serotypes: Ia, Ib, II, III and V to the HT-29 and A-431 cell lines.

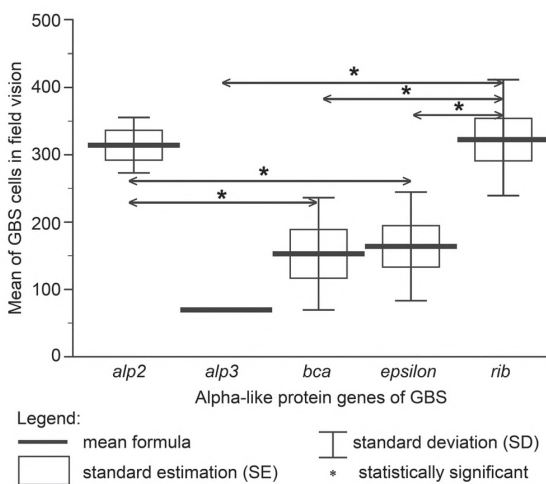


Fig. 4. The comparison of adherence of *S. agalactiae* strains in relation to the alpha-like protein genes to the HT-29 and A-431 cell lines. To assess the adherence described in this work the sole GBS strain possessing the *alp3* gene was selected.

cell lines between GBS strains of the serotypes of Ia and III ($P < 0.05$) (Fig. 5). Statistically significant differences were also confirmed between GBS strains possessing the *rib* gene and GBS strains with the following genes: *alp3*, *bca* and *epsilon* as well as between GBS strains possessing the *alp2* gene and GBS strains with the *epsilon* and *bca* genes.

The presented analysis of results confirmed statistically significant higher adherence capacities of GBS to the HT-29 cell line corresponding to the properties of the colonic epithelium than to the A-431 cells representing the model of the vaginal epithelium ($P = 0.0005$). GBS strains of the following serotypes: Ia ($P = 0.0462$) and III ($P = 0.0270$) differed in adherence

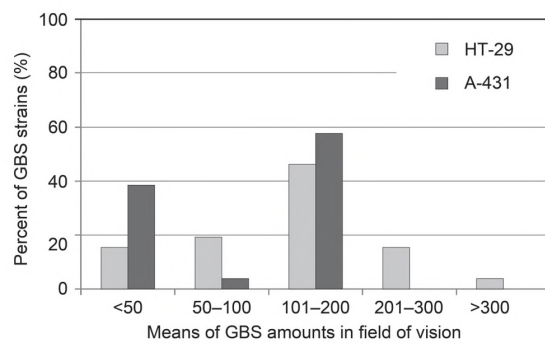


Fig. 5. The comparison of adherence of GBS between the HT-29 and A-431 cell lines.

capacities between the HT-29 and A-431 cell lines. GBS strains possessing the *rib* gene displayed differences in adherence capacities to the HT-29 and A-431 cell lines from GBS strains with the *alp2*, *alp3*, *bca* and *epsilon* genes ($P = 0.0102$).

Analysis of the adherence results of the selected GBS strains to the A-431 cell line confirmed the relation between adherence capacities of GBS strains and capsular polysaccharides ($P = 0.0355$) as well as alpha-like protein genes ($P = 0.0013$). Statistically significant differences were demonstrated between GBS strains of serotype III which displayed the highest adherence capacities and GBS strains belonging to serotype Ia which displayed the lowest adherence to both cell lines ($P < 0.05$). GBS strains possessing the *alp2* and *rib* genes displayed higher adherence to the A-431 cell line unlike GBS strains with the *alp3* gene.

On the other hand, adherence of the selected GBS strains to the HT-29 cell line depended solely on the alpha-like protein genes ($P = 0.0373$). GBS strains possessing the *alp2* and *rib* genes displayed higher adherence capacities to the HT-29 cell lines than GBS strains with the *epsilon* and *bca* genes ($P < 0.05$).

Analysis of the relationship between bacterial adhesion and capsular adhesins is very important to understanding of the infection process. All the GBS strains selected for the experiment adhered both to the HT-29 cell line corresponding to the properties of the colonic epithelium as well as to the A-431 cell line representing the model of the vaginal epithelium.

The observed adherence capacities of GBS are strains attributes so they indicate immense diversity among the analysed feature. The obtained results of GBS adherence for statistical analysis were averaged and then compared between investigated groups depending on serotype and presence of alpha-like protein genes. Presumably, adherence capacities of the examined GBS strains stemmed from the fact that they had been isolated from clinical materials of human origin. Similarly, Strus et al. (2001) examined the adherence of *Lactobacillus* strains

isolated from the human flora to the A-431 and Caco-2 cell lines and they confirmed adherence capacities in most analyzed strains.

The observed higher adherence of GBS to the HT-29 cell line than to the A-431 cell line can be accounted by the fact that GBS colonizes mainly the lower part of the gastrointestinal tract and vaginal colonization is a result of this process. In addition, this relation may result from differences in the structure of intestinal epithelial cells and vaginal epithelial cells as well as the presence of various receptors characteristic for a specific tissue (Kim *et al.*, 2010). This can be confirmed by the fact that the relation between GBS adherence to the A-431 cell line and serotype as well as the alpha-like protein genes has been observed whereas for GBS adherence to the HT-29 cell line its sole dependence on the alpha-like protein genes has been noted.

The results of numerous examinations consisting in, among other things, preliminary effects of trypsin on GBS cells, application of antibodies against specific GBS adhesins or heat treatment of GBS at the temperature of 65°C for the period of 30 minutes resulted in a considerable decrease in bacterial adherence to the tissues in comparison to the strains which did not undergo the above-mentioned processes which would suggest an important role of proteins in the process of adhesion (Tamura *et al.*, 1994). The results obtained in this work confirm the relation between the adherence of GBS strains to the A-431 and HT-29 cell lines and the presence of specific genes encoding the alpha-like proteins with particular attention to GBS strains possessing the *rib* gene. However, there are scientific reports describing a significant role of another alpha-like protein – Alpha C protein – in the process of adherence. The Alpha C protein binds glycosaminoglycan on the surface of the host cells, which consequently allows the bacteria to penetrate into the epithelial cells. However other data indicated that GBS lacking *bca* gene was not affected in adhesion to epithelial cells (Baron *et al.*, 2004; Bolduc *et al.*, 2002). To assess the adherence described in this work the sole GBS strain possessing the *alp 3* gene was selected due to the lack of GBS strains with this gene.

Perhaps alpha-like proteins play a helpful role in GBS adherence characteristic for a strain, because individual alpha-like proteins are not present in all GBS strains. Other GBS surface adhesins binding specific extracellular matrix (ECM) proteins are, among others, fibrinogen-binding proteins (FbsA and FbsB), laminin-binding proteins (Lmb), serine-rich repeat proteins, peptidase (SepB) and fimbriae (Devi *et al.*, 2010; Gutekunst *et al.*, 2004; Tannenbaum *et al.*, 2007; Samen *et al.*, 2007).

Capsular polysaccharides conditioning the affinity of a specific GBS strain to serotype probably do not have a direct influence on its adherence properties. However,

it is speculated that there can be specific adhesion proteins in a given serological type. Presumably, this could justify the relation between GBS adherence to the cell lines, capsular polysaccharides conditioning the affinity of the GBS strain to the specific serotype. For instance, among GBS strains of serotype III which is most frequently described as an etiological factor of neonatal infections, the restriction digested pattern type III strains of GBS which possess additional glycosylated proteins of adhesin-like functions were characterized (Seifert *et al.*, 2006; Anderson *et al.*, 2003).

The results of the presented experiment confirm the interesting relation between the adherence of *S. agalactiae* to the A-431 and HT-29 cell lines displaying the properties, respectively, of the vaginal epithelium as well as colonic epithelium and serotypes as well as the alpha-like protein genes. As a result, assessment of the adherence of GBS requires continuation consisting in examining more strains of *S. agalactiae* and including other tissue models in the research. Discovering the interaction which takes place between GBS cells and the epithelial cells of the host which condition the adherence as well as describing the adhesins and receptor proteins participating in this process would provide extremely valuable information. The obtained information could be used in the research on eradication of GBS from the genital tract as well as gastrointestinal tract of the pregnant women providing the protection for the baby against the infection.

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